(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 August 2005 (25.08.2005)

PCT

(10) International Publication Number
WO 2005/077333 A2

(51) International Patent Classification[7]: A61K 9/00

(21) International Application Number: PCT/US2005/004146

(22) International Filing Date: 10 February 2005 (10.02.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/543,508 10 February 2004 (10.02.2004) US

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(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** GEL-BASED DELIVERY OF RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

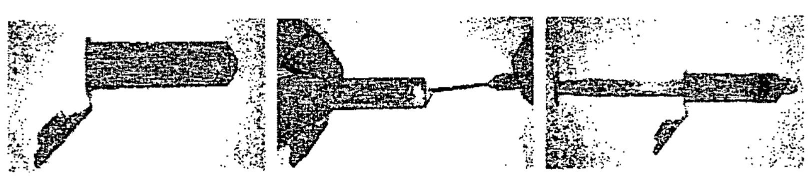

FIG. 1A *FIG. 1B* *FIG. 1C*

(57) **Abstract:** Disclosed are water-soluble gel-based compositions for the delivery of recombinant adeno-associated virus (rAAV) vectors that express nucleic acid segments encoding therapeutic constructs including peptides, polypeptides, ribozymes, and catalytic RNA molecules, to selected cells and tissues of vertebrate animals. Also disclosed are gel-based rAAV compositions are useful in the treatment of mammalian, and in particular, human diseases, including for example, cardiac disease or dysfunction, and musculoskeletal disorders and congenital myopathies, including, for example, muscular dystrophy, acid maltase deficiency (Pompe's disease), and the like. In illustrative embodiments, the invention provides rAAV vectors comprised within a biocompatible gel composition for enhanced viral delivery/transfection to mammalian tissues, and in particular to vertebrate muscle tissues such as a human heart or diaphragm tissue.

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DESCRIPTION

GEL-BASED DELIVERY OF RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

1.0 BACKGROUND OF THE INVENTION

The present application claims priority to United States Provisional Application Serial No. 60/543,508, filed February 10, 2004, the entire contents of which is specifically incorporated herein by reference. The United States government has certain rights in the present invention pursuant to grant NIDDK P01 DK58327-03 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to water-soluble gel-based compositions for the delivery of recombinant adeno-associated virus (rAAV) vectors express nucleic acid segments encoding therapeutic constructs including peptides, polypeptides, ribozymes, and catalytic RNA molecules, to selected cells and tissues of vertebrate animals. In particular, these gel-based rAAV compositions are useful in the treatment of mammalian, and in particular, human diseases, disorders, and dysfunctions. In illustrative embodiments, the invention concerns the use of rAAV vectors comprised within a gel suspension for delivery to mammalian tissues, and in particular muscle tissues of the vertebrate diaphragm. These gel-based rAAV compositions may be utilized in a variety of investigative, diagnostic and therapeutic regimens, including the prevention and treatment of musculoskeletal disorders and congenital myopathies including, for example muscular dystrophy and the like. Methods and compositions are provided for preparing gel-based rAAV vector compositions for use in the preparation of medicaments useful in central and targeted gene therapy of diseases, disorders, and dysfunctions in an animal, and in humans in particular.

1.2 DESCRIPTION OF THE RELATED ART

Previous studies of gene transfer to the diaphragm in rodents have been attempted *via* delivery of non-viral or adenoviral gene transfer vectors. Liu *et al.* (2001) recently described a method for systemic delivery of plasmid DNA carrying the full-length

dystrophin gene with subsequent targeting to the diaphragm in *mdx* mice, a mouse strain with X-linked muscular dystrophy that mimics the diaphragmatic degeneration observed in Duchenne muscular dystrophy (Stedman *et al*, 1991). In that study, which used no carrier molecules, plasmid DNA was delivered intravenously *via* tail vein followed by transient (8-second) occlusion of the vena cava at the level of the diaphragm. High levels of gene expression were measured in diaphragm homogenates the next day and for 180 days (Liu *et al.*, 2001), implicating dwell time as potentially the most significant determinant of successful gene transfer to the diaphragm with naked DNA. Two reports (Petrof *et al.*, 1995; Yang *et al.*, 1998) also indicated successful direct injection of recombinant adenoviruses carrying a mini-dystrophin gene to the diaphragms of *mdx* mice. Both studies demonstrated high levels of expression focally, presumably due to the delivery method. Transient gene expression, due to vector-related, dose-dependent inflammation, made assessment of the uniformity of gene expression difficult, but even with focal expression the authors observed measurable improvements in contractile function. More recently, Sakamoto *et al.* (2002) have developed an *mdx* strain that is transgenic for a micro-dystrophin construct, which is within the packaging capacity of rAAV.

1.3 DEFICIENCIES IN THE PRIOR ART

Currently, there are limited pharmacological approaches to providing sufficiently high titers of rAAV particles to certain cells and tissues in affected mammals. A major hurdle in most current human gene therapy strategies is the ability to transduce target tissues at very high efficiencies that ultimately lead to therapeutic levels of transgene expression. This is particular true for tissues such as the vertebrate diaphragm.

Many such methods introduce undesirable side-effects, and do not overcome the problems associated with traditional modalities and treatment regimens for such conditions. Thus, the need exists for an effective treatment that circumvents the adverse effects and provides more desirable results, with longer acting effects, and improved compliance in both human and veterinary patients.

2.0 SUMMARY OF THE INVENTION

The present invention overcomes these and other limitations inherent in the prior art by providing a new gel-based method for delivery of recombinant adeno-associated virus (AAV) vectors. In illustrative embodiments of this new system, recombinant AAV vectors

are mixed with a water-soluble glycerin-based gel and applied directly to the target tissue. The gel provides increased exposure time of target cells to the vector, thereby increasing the efficiency of transduction in the targeted areas.

In one embodiment, the invention discloses and claims a composition comprising a recombinant adeno-associated viral vector and a water-soluble biocompatible gel. The rAAV vector may comprise rAAV virions, or rAAV particles, or pluralities thereof. Preferably the gel comprises a matrix, a hydrogel, or a polymer, which may optionally be cross-linked, stabilized, chemically conjugated, or otherwise modified. The gel may optionally be a sustained release formulation, or may be biodegradable. Such gels may comprise one or more polymers, viscous contrast agents (such as iodixanol) or other viscosity- or density-enhancing agents, including for example, polysaccharides, including sucrose-based media (e.g., sucrose acetate isobutyrate).

The composition may comprise a biocompatible gel such as one or more of the commercially-available gel compounds including for example, SAF-Gel, Duoderm Hydroactive Gel, Nu-Gel; Carrasyn (V) Acemannan Hydrogel, Elta Hydrogel or K-Y Sterile Gel.

In preferred embodiments, the gel comprises glycerin, gelatin, or alginate, or derivatives, mixtures, or combinations thereof. In typical formulations developed for administration to a mammal, and particularly for compositions formulated for human administration, the gel may comprise substantially all of the non-viral weight of the composition, and may comprise as much as about 98% or 99% of the composition by weight. This is particular desirous when substantially non-fluid, or substantially viscous formulations of the rAAV particles, vectors, or virions are preferred. When slightly less viscous, or slightly more fluid compositions are desired, the biocompatible gel portion of the composition may comprise at least about 50% by weight, at least about 60% by weight, at least about 70% by weight, or even at least about 80% or 90% by weight of the composition. Of course, all intermediate integers within these ranges are contemplated to fall within the scope of this disclosure, and in certain embodiments, even more fluid (and consequently less viscous) gel/viral compositions may be formulated, such as for example, those in which the gel or matrix component of the mixture comprises not more than about 50% by weight, not more than about 40% by weight, not more than about 30% by weight, or even those than comprise not more than about 15% or 20% by weight of the composition

In such exemplary formulations, the recombinant adeno-associated viral vectors may comprise either wild-type or genetically-modified rAAV vectors, including for example, recombinant vectors obtained from an AAV serotype 1 strain (rAAV1), an AAV serotype 2 strain (rAAV2), an AAV serotype 3 strain (rAAV3), an AAV serotype 4 strain (rAAV4), an AAV serotype 5 strain (rAAV5), an AAV serotype 6 strain (rAAV6), an AAV serotype 7 strain (rAAV7), an AAV serotype 8 strain (rAAV8), or an AAV serotype 9 strain (rAAV9), or combinations of two or more of such vectors. Optionally the composition may comprise a second viral or non-viral vector, or other therapeutic component as deemed necessary for the particular application. Such viral vectors may include, but are not limited to, Adenoviral vectors (AV), Herpes simplex virus vectors (HSV), and others such like that are known in the art.

Preferably, in almost all cases, the recombinant adeno-associated viral vectors formulated in the biocompatible water-soluble gels and matrices disclosed here will comprise at least a first nucleic acid segment that encodes one or more therapeutic agents, and that is expressed in a mammalian cell suitably comprising the rAAV vector. Such therapeutic agents may comprise one or more nucleic acids, peptides, polypeptides, proteins, antibodies, antigens, epitopes, binding domains, antisense molecules, or catalytic RNA molecules (such as, for example, a hammerhead or hairpin ribozyme having specificity for a target polynucleotide within the selected host cells into which the rAAV compositions are delivered and/or expressed.

In certain embodiments, the gel compositions my further optionally comprise one or more pharmaceutical excipients, diluents, buffers, or such like, and may further comprise one or more lipid complexes, liposomes, nanocapsules, microspheres, or other agents which may enhance, stabilize, or facilitate uptake of the rAAV vectors by suitable cells or tissue types either *in vitro* or *ex vivo*, or within the body of the animal into which the rAAV vector compositions are introduced (*in situ* and *in vivo*).

In important embodiments, the compositions of the present invention are formulated and intended for use in therapy, particularly in the therapy of mammals, including humans, domesticated livestock, and animals under the care of a veterinarian or other trained animal medicine practitioner, that have, are suspected of having, or are at risk for developing one or more diseases, disorders, or dysfunctions, including for example, musculoskeletal diseases and congenital myopathies, such as muscular dystrophy and related conditions.

The invention also provides kits for diagnosing, preventing, treating or ameliorating the symptoms of a diseases or disorder in a mammal. Such kits generally will comprise one or more of the water-soluble gell-based rAAV compositions as disclosed herein, and instructions for using said kit.

The invention also contemplates the use of one or more of the disclosed compositions, in the manufacture of medicaments for treating, abating, reducing, or ameliorating the symptoms of a disease, dysfunction, or disorder in a mammal, such as a human that has, is suspected of having, or at risk for developing a musculoskeletal disorder or a congenital myopathy such as muscular dystrophy.

The invention also contemplates the use of one or more of the disclosed compositions, in the manufacture of compositions and/or medicaments for increasing the bioavailability, cellular binding, cellular uptake, or increasing or altering the tissue-specificity for a particular AAV-derived vector used in a particular animal or cell type. The compositions of the invention are contemplated to be particularly useful in improving the transformation efficiency, and/or increasing the titer of a particular rAAV vector for a given cell, tissue, or organ into which introduction of rAAV vectors is desired. The inventors have demonstrated that the use of the disclosed gel-based delivery vehicles can substantially improve the efficiency of transformation for various cell and/or tissue types. As such, the compositions disclosed herein are particularly useful in providing a means for improving cellular uptake or viral infectivity of a given cell or tissue type.

Methods are also provided by the present invention for administering to a mammal in need thereof, an effective amount of at least a first therapeutic agent in an amount and for a time sufficient to provide the mammal with one or more of the disclosed compositions *via* introduction of such compositions into suitable cells or tissues of the mammal, either *in vitro*, *in vivo*, *in situ*, or *ex situ*. Such methods are particularly desirable in the treatment, amelioration, or prevention of diseases, including myopathies such as muscular dystrophy and the like, for which the inventors contemplate that administration of sufficiently high titers of suitable therapeutic rAAV gel-based compositions directly into the diaphragm of affected individuals will afford expression of one or more suitable therapeutic agents necessary to facilitate treatment.

In these and all other therapeutic embodiments, the rAAV compositions may be introduced into cells or tissues by any means suitable, including for example, by systemic or localized injection, or by other means of viral delivery as may be known in the art,

including, but not limited to topical, intravenous, intramuscular, intraorgan, or transabdominal delivery, or other means such as transdermal administration.

In one embodiment, the present invention provides for a composition that comprises, consists essentially of, or consists of: a recombinant adeno-associated viral vector that comprises a nucleic acid segment that encodes a mammalian therapeutic agent; and a water-soluble biocompatible gel, gel matrix, sol, or sol matrix.

Such biocompatible gels, sols and matrices may comprise, consist essentially of, or consist of a biogel, a hydrogel, a polymer, a monosaccharide, a polysaccharide, an oligosaccharide, or a viscosity agent. Exemplary viscosity agents include viscous contrast agents such as iodixanol, or a saccharide-containing component such as a fructose, sucrose, lactose, glucose, or arabinose-containing compound.

In illustrative embodiments, the biocompatible gel may comprise, consist essentially of, or consist of glycerin or a glycerin-derived compound, a gelatin or a gelatin-derived compound, or an alginate or an alginate-derived compound. Exemplary biocompatible gels which are commercially available include, but are not limited to, SAF-Gel, Duoderm Hydroactive Gel, Nu-Gel; Carrasyn (V) Acemannan Hydrogel, Elta Hydrogel and K-Y Sterile Gel, to name only a few. The inventors contemplate virtually any gel or matrix material will be useful in the practice of the invention so long as it is not deleterious to the mammalian host cells into which it is introduced, or to the particular viral particles or virions which are suspended in the gel. In some instances, it may be desirable to use a plurality of two or more different gel materials to formulation the composition. One or more of such biocompatible gels may be partially, or substantially entirely cross-linked by one or more cross-linking agents. Alternatively, one or more of such biocompatible gels may be partially, or substantially entirely conjugated to one or more additional molecules, such as dyes, ligands, carriers, liposomes, lipoproteins, or other chemical or pharmaceutical compounds.

Preferably in the practice of the invention, the number of viral vectors, viral particles, and/or virions comprised within the biocompatible gel will be at least on the order of about 1 or 2×10^{11} AAV particles per milliliter, and more preferably on the order of about 3 or 4×10^{11} AAV particles per milliliter, and more preferably still, on the order of about 7 or 8×10^{11} AAV particles per milliliter. Alternatively, when a higher titer of particles is desired, the compositions of the present invention may comprise about 1×10^{12} AAV particles per milliliter, 2×10^{12} AAV particles per milliliter, 5×10^{12} AAV particles

per milliliter, 7×10^{12} AAV particles per milliliter, or even about 1×10^{13} AAV particles per milliliter, 3×10^{13} AAV particles per milliliter, or 5×10^{13} or so AAV particles per milliliter.

In the practice of the invention, the biocompatible gel may comprise at least about 50% by weight of the composition, at least about 55%, or at least about 60% by weight of the composition. In other embodiments, when an even more viscous medium is preferred, the biocompatible gel may comprise at least about 65%, at least about 70%, at least about 75%, or even at least about 80% or so by weight of the composition. In highly concentrated samples, the biocompatible gel may comprise at least about 85%, at least about 90% or at least about 95% or more by weight of the viral composition. The compositions may optionally also comprise one or more biological diluents or buffers, or some other pharmaceutically-acceptable vehicle or excipient.

The mammalian therapeutic agents used in the practice of the invention may be a nucleic acid segment that encodes a mammalian peptide, polypeptide, enzyme, or protein, or alternatively, may comprise a polynucleotide sequence that encodes either an antisense or a catalytic RNA molecule (ribozyme).

Preferably, the mammalian therapeutic agent is a peptide, polypeptide, enzyme, protein, antisense, or ribozyme that can be expressed in one or more human tissues, and particularly in human muscle tissues, such as diaphragm and cardiac muscle tissues.

Examples of mammalian therapeutic agents contemplated for use in the present invention are those agents that treat, prevent, or ameliorate the symptoms of one or more muscular, neuromuscular, myopathic, or neuropathic diseases, disorders, dysfunctions or abnormalities. Examples of such polypeptides include, but are not limited to, biologically-active mammalian (and particularly human) acid α-glucosidase (GAA), dystrophin, or α-1 antitrypsin polypeptides.

The invention also provides therapeutic and diagnostic kits that typically comprise one or more of the AAV gel-based compositions and instructions for using the kit in particular regimens or modalities.

The invention also provides uses of the compositions in a method for providing a biologically-effective amount of a therapeutic agent to a tissue site of a mammal in need thereof. The method generally involves at least the step of providing one or more of the disclosed AAV gel-based therapeutic compositions to a mammal in need thereof in an amount and for a time effective to provide a biologically-effective amount of the encoded therapeutic agent to particular cells, tissues, or organ(s) of the animal being treated.

Typical modes of administration of the compositions include, for example, transfection, systemic administration, or by direct, indirect, or localized injection to a cell, tissue, or organ of the mammal using methodologies that are routine to those practicing in the related art. In preferred embodiments, the mammal is a human that has, is suspected of having, or at risk for developing a musculoskeletal disorder, a glycogen storage disease, a neuromuscular disorder, a neuropathic condition, or a congenital myopathy, injury, or trauma. Exemplary conditions for which treatment using one of more of the disclosed AAV compositions is highly preferred include, for example, muscular dystrophy (including, for example, the Duchenne Becker form), cardiac injury, infart, trauma, ischemia, or hypertrophy, or metabolic disorders such as acid maltase deficiency (also known as Pompe's Disease).

The invention also provides for uses of the compositions in a method for treating or preventing a musculoskeletal disease or dysfunction, or a congenital myopathy in a mammal. The method generally involves at least the step of providing to such a mammal, one or more of the AAV gel-based compositions, in an amount and for a time effective to treat or prevent the musculoskeletal disease or dysfunction, or congenital myopathy in the animal. In preferred embodiments, the mammal is a human that has, is suspected of having, or is at risk for developing musculoskeletal disease or congenital myopathy.

In another embodiment, the invention provides for uses of the disclosed AAV gel-based compositions in a method of expressing in cells of a mammalian heart or diaphragm muscle, a nucleic acid segment that encodes an exogenously-provided mammalian therapeutic agent. In an overall and general sense, the method generally comprises at least the step of injecting into heart or diaphragm tissue, one or more of the disclosed AAV-therapeutic gene constructs in an amount and for a time effective to express the exogenously-provided mammalian therapeutic agent.

The invention also provides in another embodiment, a use for the disclosed AAV gel-based compositions in a method for treating or ameliorating the symptoms of a congenital myopathy in a mammal. This method generally comprises administering to a mammal in need thereof, one or more of the disclosed AAV-therapeutic gene constructs, in an amount and for a time sufficient to treat or ameliorate the symptoms of the congenital myopathy in the mammal.

Also disclosed are methods and compositions for expressing a biologically-effective amount of an exogenously-supplied therapeutic polynucleotide construct that encodes a therapeutic agent such as a peptide, polypeptide or protein in a mammalian diaphragm, heart, or muscle cell. The method generally involves: introducing into a population of mammalian diaphragm, heart, or muscle cells, an amount of an AAV gel-based composition, for a time effective to express a biologically-effective amount of the exogenously-supplied therapeutic agent in the cells that were transfected with he composition and that express the heterologous gene to produce the encoded polypeptide product in the diaphragm, heart or muscle cells.

In such methods, the therapeutic peptide, polypeptide or protein may be an antibody, an antigenic fragment, an enzyme, a kinase, a protease, a glucosidase (including for example human acid alpha- and beta-glucosidases), a glycosidase (including for example human acid alpha- and beta-glycosidases), a nuclease, a growth factor, a tissue factor, a myogenic factor, a neurotrophic factor, a neurotrophin, a dystrophin, an interleukin, or a cytokine.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1A, **FIG. 1B** and **FIG. 1C** show an illustrative gel-based delivery preparation. FIG. 1A shows rAAV vectors mixed in a 2-mL microcentrifuge tube and then centrifuged briefly. FIG. 2B shows the tube is punctured using a 22-gauge needle, creating an aperture through which the virus-gel suspension can be propelled. FIG. 1C shows a plunger from a standard 3 cc syringe is used to push the vector from the tube, enabling its application to the diaphragmatic surface. The oblique, bottom surface of the microcentrifuge tube is used to distribute the vector-gel suspension evenly on the surface.

FIG. 2A and **FIG. 2B** show free virus and gel-based delivery of rAAV-βgal vectors based on AAV serotypes 1, 2, and 5. Adult wild-type mice (129X1×C57BL/6) were treated with 1×10^{11} particles of rAAV-βgal, with virus either directly applied to the diaphragm or applied using the gel-based method. The animals were sacrificed six weeks later and tissues

were collected and assayed for β-galactosidase activity. FIG. 2A shows representative histochemical (X-gal) stained diaphragm segments from treated animals. Each row corresponds to the respective serotype into which the recombinant vector genome was packaged (AAV1, 2, and 5, respectively). The columns represent application of free virus (left) or virus-gel suspension (right) to the abdominal surface of the diaphragms, respectively. Note the intense blue staining in both columns for vector virions packaged using the rAAV1 capsid (top row), with increased intensity using the gel-based method (top row, right panel). FIG. 2B shows quantitative assay of β-galactosidase activity from the same animals. The bars represent mean ± SEM GAA activity for three mice in each group.

FIG. 3A and **FIG. 3B** show an illustrative embodiment of the invention in which rAAV1-hGAA-mediated transduction of the diaphragms of *Gaa*$^{-/-}$ mice was demonstrated. FIG. 3A shows adult *Gaa*$^{-/-}$ mice were treated with 1×10^{11} particles of rAAV1-*GAA* in the quadriceps muscle. Wild-type (wt) and untreated *Gaa*$^{-/-}$ (mock) mice were used as controls. Muscle tissues were isolated at 6 weeks after treatment and assayed for GAA activity. The bars represent mean ± SEM GAA activity for three mice in each group. FIG. 3B shows representative sections of sections from free vector- (left) and gel-based vector-treated (right) *Gaa*$^{-/-}$ mouse diaphragms, stained for glycogen using periodic acid-Schiff's reagent. Glycogen-containing vacuoles and regions acquire a pink stain using this technique.

FIG. 4 shows biodistribution of rAAV1 vector genomes after gel-based delivery. Nested PCR™ was used to amplify AAV genomes carrying the β-galactosidase gene after isolating tissues from gel-based rAAV1-βgal treated mice. Total cellular DNA was extracted and AAV genomes were amplified using primers specific for the βgal transgene. The expected product is 333 bp, and the positive control is the vector plasmid that was used to package the rAAV particles.

FIG. 5 is a graph showing conditional GAA expression in Mck-T-GAA/Gaa$^{-/-}$ mice.

FIG. 6 is a graph showing GAA activity post intramyocardial injection of AAV.

FIG. 7 is a graph showing GAA activity after neonatal IV delivery.

FIG. 8 shows PAS of heart tissue.

FIG. 9 is a graph showing soleus contractile force.

FIG. 10 is a graph showing *LacZ* expression after neonatal intracardiac delivery.

4.0 Description of Illustrative Embodiments

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

Mouse models of human disease provide invaluable opportunities to evaluate the potential efficacy of candidate therapies. Gene therapy strategies in particular have benefited enormously from the profusion of knockout and transgenic mice that recapitulate the genetic and pathophysiologic features of human diseases. Congenital myopathies, including the muscular dystrophies, have been widely investigated as targets for gene therapy interventions, and the diaphragm is often cited as one of the important target organs for functional correction (Petrof, 1998).

The mouse diaphragm presents unique challenges in terms of delivery of therapeutic agents due to its small size and thickness, which preclude direct injection into the muscle. Intravenous or intra-arterial delivery of vectors have not yet proven to be effective alternatives, but some studies are nevertheless currently under investigation (Baranov *et al.*, 1999; Liu *et al.*, 2001). However, isolation of blood vessels that specifically perfuse the diaphragm is also difficult in the mouse. Systemic delivery of vectors may eventually require the application of capsid-based targeting methods that have recently been reported (Buning *et al.*, 2003; Muller *et al.*, 2003; Perabo *et al.*, 2003; Ponnazhagan *et al.*, 2002; Shi *et al.*, 2001; Shi and Bartlett, 2003; Wu *et al.*, 2000).

4.1 Adeno-Associated Virus

Adeno-associated virus is a single-stranded DNA-containing, non-pathogenic human parvovirus that is being widely investigated as a therapeutic vector for a host of muscle disorders (Muzyczka, 1992; Kessler *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997). Six serotypes of the virus (AAV1-6) were originally described, and two more have recently been identified in rhesus macaques (Gao *et al.*, 2002). Recombinant adeno-associated virus

(rAAV) vectors have been developed in which the *rep* and *cap* open reading frames of the wild-type virus have been completely replaced by a therapeutic or reporter gene, retaining only the characteristic inverted terminal repeats (ITRs), the sole *cis*-acting elements required for virus packaging. Using helper plasmids expressing various combinations of the AAV2 *rep* and AAV1, 2, and 5 *cap* genes, respectively, efficient cross-packaging of AAV2 genomes into particles containing the AAV1, 2, or 5 capsid protein has been demonstrated (Grimm *et al.*, 2003; Xiao *et al.*, 1999; Zolotukhin *et al.*, 2002; Rabinowitz *et al.*, 2002). The various serotype vectors have demonstrated distinct tropisms for different tissue types *in vivo*, due in part to their putative cell surface receptors. Although several reports have indicated that rAAV1 vectors efficiently transduce skeletal muscle in general (Fraites *et al.*, 2002; Chao *et al.*, 2001; Hauck and Xiao, 2003), no study to date has reported which of the serotypes, if any, might transduce the diaphragm in particular.

4.2 PROMOTERS AND ENHANCERS

Recombinant vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate a therapeutic polypeptide product from a transcribed gene that is comprised within one or more of the rAAV compositions disclosed herein.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively linked," "operably linked," "operatively positioned," "under the control of" or "under the transcriptional control of" means that the promoter is in the correct location and orientation in relation to the nucleic acid segment that comprises the therapeutic gene to properly facilitate, control, or regulate RNA polymerase initiation and expression of the therapeutic gene to produce the therapeutic peptide, polypeptide, ribozyme, or antisense RNA molecule in the cells that comprise and express the genetic construct.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the therapeutic agent-encoding polynucleotide segment under the control of

one or more recombinant, or heterologous, promoter(s). As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with the particular therapeutic gene of interest in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the therapeutic agent-encoding nucleic acid segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a mammalian, bacterial, fungal, or viral promoter. Exemplary such promoters include, for example, a β-actin promoter, a native or modified CMV promoter, an AV or modified AV

promoter, or an HSV or modified HSV promoter. In certain aspects of the invention, inducible promoters, such as tetracycline-controlled promoters, are also contemplated to be useful in certain cell types.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 below list several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of the therapeutic polypeptide-encoding rAAV constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Orntz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987a
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_{1}-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989

PROMOTER/ENHANCER	REFERENCES
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a therapeutic agent, such as a therapeutic peptide, polypeptide, ribozyme, or catalytic mRNA molecule has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly

introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a therapeutic gene in accordance with the present invention one would prepare an rAAV expression vector that comprises a therapeutic peptide- polypeptide- ribozyme- or antisense mRNA-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context. Particularly preferred recombinant vector constructs are those that comprise an rAAV vector comprised within the novel gel-based pharmaceutical vehicles disclosed herein. Such vectors are described in detail herein, and are also described in detail in U. S. Patents 6,146,874, and 6,461,606; U. S. Pat. Appl. Publ. Nos. US2003/0095949, US2003/0082162; and PCT Intl. Pat. Appl. Publ. Nos. PCT/US99/11945, PCT/US99/21681, PCT/US98/08003, PCT/US98/07968, PCT/US99/08921, PCT/US99/22052, PCT/US00/11509, PCT/US02/13679, PCT/US03/13583, PCT/US03/13592, PCT/US03/08667, PCT/US03/20746, PCT/US03/12324, and PCT/US03/12225 (each of which is commonly owned with the present application, and is specifically incorporated herein by reference in its entirety).

4.3 PHARMACEUTICAL COMPOSITIONS

In certain embodiments, the present invention concerns formulation of one or more of the rAAV compositions disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. In particular, the present invention contemplates the formulation of one or more rAAV vectors, virions, or virus particles (or pluralities thereof) using a water-soluble glycerin-based gel.

In such pharmaceutical compositions, it will also be understood that, if desired, the rAAV-encoded nucleic acid segment, RNA, DNA or PNA compositions that express one or more therapeutic gene product(s) as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, peptides, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or topical administrations of

the gel-based rAAV vector formulations described herein. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The rAAV compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein.. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, topical, sublingual, subcutaneous, transdermal, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

In typical application, the water-soluble glycerin-based gel formulations utilized in the preparation of pharmaceutical delivery vehicles that comprise one or more rAAV constructs may contain at least about 0.1% of the water-soluble glycerin compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1% and about 95% or more preferably, between about 5% and about 80%, and stil more preferably, between about 10% and about 60% or more of the weight or volume of the total pharmaceutical rAAV formulation, although the inventors contemplate any concentrations within those ranges may be useful in particular formulations. Naturally, the amount of the gel compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

Owing to particular gel's characteristics, (from extremely viscous to almost water-like) the amount of gel used in the disclosed rAAV compositions may be titrated to achieve desirable or optimal results in particular treatment regimens. Such formulations, and the determination of the appropriate gel and concentration to use will be within the abilities of the artisan skilled in this field having benefit of the present teachings.

While the embodiments presented herein have specifically incorporated water-soluble glycerin gels, other gel compositions are also contemplated to be useful depending upon the particular embodiment, and as such are considered to fall within the scope of the present disclosure. For example, other currently commercially-available glycerin-based gels, glycerin-derived compounds, conjugated, or crosslinked gels, matrices, hydrogels, and polymers, as well as gelatins and their derivatives, alginates, and alginate-based gels, and even various native and synthetic hydrogel and hydrogel-derived compounds are all expected to be useful in the formulation of various rAAV pharmaceutical compositions. Specifically, illustrative embodiment gels include, but are not limited to, alginate hydrogels SAF-Gel (ConvaTec, Princeton, NJ), Duoderm Hydroactive Gel (ConvaTec), Nu-gel (Johnson & Johnson Medical, Arlington, TX); Carrasyn (V) Acemannan Hydrogel (Carrington Laboratories, Inc., Irving, TX); glycerin gels Elta Hydrogel (Swiss-American Products, Inc., Dallas, TX) and K-Y Sterile (Johnson & Johnson). In addition, viscous contrast agents such as iodixanol (Visipaque, Amersham Health), and sucrose-based mediums like sucrose acetate isobutyrate (SAIB) (Eastman Chemical Company, Kingsport, TN) are also contemplated to be useful in certain embodiments. Additionally, biodegradable biocompatible gels may also represent compounds present in certain of the rAAV formulations disclosed and described herein.

In certain embodiments, a single gel formulation may be used, in which one or more rAAV compositions may be present, while in other embodiments, it may be desirable to form a pharmaceutical composition that comprises a mixture of two or more distinct gel formulations may be used, in which one or more rAAV particles, virus, or virions are present. Various combinations of sols, gels and/or biocompatible matrices may also be employed to provide particularly desirable characteristics of certain viral formulations. In certain instances, the gel compositions may be cross-linked by one or more agents to alter or improve the properties of the virus/gel composition.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures

thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a mammal, and in particular, when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid

prior to injection can also be prepared. The preparation can also be emulsified. In certain embodiments, the rAAV-gel compositions of the present invention may be formulated for topical, or transdermal delivery to one or more tissue sites or cell types within the body of the vertebrate being treated. Alternatively, in the embodiments where *ex vivo* or *ex situ* modalities are preferred, the rAAV-gel compositions of the invention my be used externally from the body of the intended recipient by first contacting a cell suspension or a tissue sample, or other extracorporeal composition with the rAAV-gel compositions to facilitate transfer of the rAAV vectors into the cells or tissues in ex vivo fashion. Following suitable transfection, then, such cells or tissues could be reintroduced into the body of the animal being treated.

4.4 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

In certain embodiments, the rAAV-gel based compositions of the present invention may further comprise one or more liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for enhancing, facilitating, or increasing the effectiveness of introducing the therapeutic rAAV compositions of the present invention into suitable host cells, tissues, or organs. In particular, the addition of a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like to the gel-based compositions of the invention may serve to enhance or facilitate the delivery of the rAAV vectors, virions, or virus particles into the target cells or tissues.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985a; 1985b; Coune, 1988; Sculier *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1980), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as

the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues

for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.5 THERAPEUTIC AND DIAGNOSTIC KITS

The invention also encompasses one or more compositions together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular rAAV-polynucleotide delivery formulations, and in the preparation of therapeutic agents for administration to a mammal, and in particularly, to a human, for one or more of the indications described herein for which rAAV-based gene therapy provides an alternative to current treatment modalities. In particular, such kits may comprise one or more gel-based rAAV composition in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, porcines, canines, and felines. The composition may include partially or significantly purified rAAV compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed water-soluble gel-based rAAV composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of therapeutic compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.,* injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.6 METHODS OF NUCLEIC ACID DELIVERY AND DNA TRANSFECTION

In certain embodiments, it is contemplated that one or more of the rAAV-delivered therapeutic product-encoding RNA, DNA, PNAs and/or substituted polynucleotide compositions disclosed herein will be used to transfect an appropriate host cell. Technology for introduction of rAAVs comprising one or more PNAs, RNAs, and DNAs into target host cells is well known to those of skill in the art.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention for use in certain *in vitro* embodiments, and under conditions where the use of rAAV-mediated delivery is less desirable. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation

(Wong and Neumann, 1982; Fromm *et al.*, 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki et al., 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.7 Expression in Animal Cells

The inventors contemplate that a polynucleotide comprising a contiguous nucleic acid sequence that encodes a therapeutic agent of the present invention may be utilized to treat one or more cellular defects in a host cell that comprises the vector. Such cells are preferably animal cells, including mammalian cells such as those obtained from a human or other primates, murine, canine, feline, ovine, caprine, bovine, equine, epine, or porcine species. In particular, the use of such constructs for the treatment and/or amelioration of one or more diseases, dysfunctions, or disorders in a human subject that has, is suspected having, or has been diagnosed with such a condition is highly contemplated. The cells may be transformed with one or more rAAV gel-based vector compositions that comprise at least a first therapeutic construct of interest, such that the genetic construct introduced into and expressed in the host cells of the animal is sufficient to treat, alter, reduce, diminish, ameliorate or prevent one or more deleterious conditions in such an animal when the composition is administered to the animal either *ex situ*, *in vitro* and/or *in vivo*.

4.8 Transgenic Animals

It is contemplated that in some instances the genome of a transgenic non-human animal of the present invention will have been altered through the stable introduction of one or more of the rAAV-delivered polynucleotide compositions described herein, either native, synthetically modified, or mutated. As used herein, the term "transgenic animal" is intended to refer to an animal that has incorporated exogenous DNA sequences into its genome. In designing a heterologous gene for expression in animals, sequences which interfere with the efficacy of gene expression, such as polyadenylation signals, polymerase II termination sequences, hairpins, consensus splice sites and the like, are eliminated. Current advances in

transgenic approaches and techniques have permitted the manipulation of a variety of animal genomes *via* gene addition, gene deletion, or gene modifications (Franz *et al.*, 1997). For example, mosquitoes (Fallon, 1996), trout (Ono *et al.*, 1997), zebrafish (Caldovic and Hackett, 1995), pigs (Van Cott *et al.*, 1997) and cows (Haskell and Bowen, 1995), are just a few of the many animals being studied by transgenics. The creation of transgenic animals that express human proteins such as α-1-antitrypsin, in sheep (Carver *et al.*, 1993); decay accelerating factor, in pigs (Cozzi *et al.*, 1997), and plasminogen activator, in goats (Ebert *et al.*, 1991) has previously been demonstrated. The transgenic synthesis of human hemoglobin (U. S. Patent 5,602,306) and fibrinogen (U. S. Patent 5,639,940) in non-human animals have also been disclosed, each specifically incorporated herein by reference in its entirety. Further, transgenic mice and rat models have recently been described as new directions to study and treat cardiovascular diseases such as hypertension in humans (Franz *et al.*, 1997; Pinto-Siestma and Paul, 1997). The construction of a transgenic mouse model has recently been used to assay potential treatments for Alzheimer's disease (U. S. Patent 5,720,936, specifically incorporated herein by reference in its entirety). It is contemplated in the present invention that transgenic animals contribute valuable information as models for studying the effects of rAAV-delivered therapeutic compositions on correcting genetic defects and treating a variety of disorders in an animal.

4.9 Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent polypeptides, through specific mutagenesis of the underlying polynucleotides that encode them. The technique, well-known to those of skill in the art, further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed rAAV constructs to alter the activity or effectiveness of such constructs in increasing or altering their therapeutic activity, or to effect higher or more desirable introduction in a particular host cell or tissue. Likewise in certain embodiments, the inventors contemplate the mutagenesis of the therapeutic genes comprised in such rAAV vector themselves, or of the rAAV delivery vehicle to facilitate improved regulation of the particular therapeutic construct's activity, solubility, stability, expression, or efficacy *in vitro, in situ*, and/or *in vivo.*

The techniques of site-specific mutagenesis are well known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector that includes within its sequence a DNA sequence that encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation that result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing. Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety.

A number of template dependent processes are available to amplify the target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR™) which is described in detail in U. S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety. Briefly, in PCR™, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase (e.g., *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products,

excess primers will bind to the target and to the reaction product and the process is repeated. Preferably reverse transcription and PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction (referred to as LCR), disclosed in Eur. Pat. Appl. Publ. No. 320,308 (specifically incorporated herein by reference in its entirety). In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U. S. Patent No. 4,883,750, incorporated herein by reference in its entirety, describes an alternative method of amplification similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Intl. Pat. Appl. Publ. No. PCT/US87/00880, incorporated herein by reference in its entirety, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]triphosphates in one strand of a restriction site (Walker *et al.*, 1992), may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids that involves multiple rounds of strand displacement and synthesis, *i.e.* nick translation. A similar method, called Repair Chain Reaction (RCR) is another method of amplification which may be useful in the present invention and is involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.

Sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having a 3' and 5' sequences of non-target DNA and an internal or "middle" sequence

of the target protein specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe are identified as distinctive products by generating a signal that is released after digestion. The original template is annealed to another cycling probe and the reaction is repeated. Thus, CPR involves amplifying a signal generated by hybridization of a probe to a target gene specific expressed nucleic acid.

Still other amplification methods described in Great Britain Pat. Appl. No. 2 202 328, and in PCT Intl. Pat. Appl. Publ. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template and enzyme dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (Kwoh *et al.*, 1989; PCT Intl. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference in its entirety), including nucleic acid sequence based amplification (NASBA) and 3SR. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer that has sequences specific to the target sequence. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat-denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target-specific primer, followed by polymerization. The double stranded DNA molecules are then multiply transcribed by a polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into DNA, and transcribed once again with a polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target-specific sequences.

Eur. Pat. Appl. Publ. No. 329,822, incorporated herein by reference in its entirety, disclose a nucleic acid amplification process involving cyclically synthesizing

single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a first template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in a duplex with either DNA or RNA). The resultant ssDNA is a second template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to its template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting as a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

PCT Intl. Pat. Appl. Publ. No. WO 89/06700, incorporated herein by reference in its entirety, disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic; i.e. new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" (Frohman, 1990), and "one-sided PCR" (Ohara *et al.,* 1989) which are well-known to those of skill in the art.

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide (Wu and Dean, 1996, incorporated herein by reference in its entirety), may also be used in the amplification of DNA sequences of the present invention.

4.10 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes may be made in the structure of the rAAV vectors or the therapeutic agents encoded by the and still obtain functional vectors, viral particles, and virion that encode one or more therapeutic agents with desirable characteristics. As mentioned above, it is often desirable to introduce one or more mutations into a specific

polynucleotide sequence. In certain circumstances, the resulting encoded polypeptide sequence is altered by this mutation, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

TABLE 3

AMINO ACIDS		CODONS						
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		

AMINO ACIDS		CODONS						
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.11 GLYCOGEN STORAGE DISEASE TYPE II (GSDII)

GSDII is an inherited disorder of glycogen metabolism, resulting from a lack of functional acid α-glucosidase (GAA), and is characterized by progressive skeletal muscle weakness (Hers, 1963; Hirschhorn and Reuser, 2000). GAA is responsible for cleaving α-1,4 and α-1,6 linkages of lysosomal glycogen, which leads to the release of monosaccharides (Hirschhorn and Reuser, 2000; Baudhuin and Hers, 1964). A deficiency of functional GAA results in massive accumulation of glycogen in lysosomal compartments of striated muscle, resulting in disruption of the contractile machinery of the cell. Affected individuals begin storing glycogen *in utero*, ultimately resulting in a variety of pathophysiological effects, most significantly of which are severe cardiomyopathy and respiratory insufficiency (Moufarrej and Bertorini, 1993). Clinical presentation of GSDII disease can occur within the first few months of life, and most affected infants do not survive past two years of age due to cardio-respiratory failure (Hers, 1963; Hirschhorn and

Reuser, 2000; Reuser *et al.*, 1995). There are no currently established treatments for GSDII disease, however enzyme replacement therapy is being tested in clinical trials.

Strict genotype-phenotype correlations have not been established due to the small population of patients and the observation that some patients with identical mutations in the *GAA* gene have markedly different clinical presentations (Anand, 2003). The existence of modifier genes has been proposed, but to-date none have been identified.

4.12 Recombinant AAV-Mediated Gene Therapy

Recombinant AAV-based gene therapy vectors are at the forefront of viral vector-based human gene therapy applications and are currently being assessed in clinical trials (Manno *et al.*, 2003; Wagner *et al.*, 2002). Advantages of rAAV vectors include the lack of any known pathologies associated with AAV infection, the ability to infect non-dividing cells, the lack of any viral genes in the vector, and the ability to persist long-term in infected cells (Berns and Linden, 1995; Berns and Giraud, 1996; Mah *et al.*, 2002; Muzyczka, 1992; Rabinowitz and Samulski, 1998). To-date, over 40 different clones of AAV have been isolated, of which serotypes 1 though 9 have been developed into gene therapy vectors (Gao *et al.*, 2003; Gao *et al.*, 2002). Recently, several studies have demonstrated alternate tissue tropisms for each AAV serotype (Chao *et al.*, 2000; Fraites *et al.*, 2003; Rutledge *et al.*, 1998; Zabner *et al.*, 2000).

Recombinant AAV-mediated gene therapy strategies have demonstrated significant promise for the treatment of GSDII and the muscular dystrophies. Preclinical studies have demonstrated phenotypic correction of a mouse model of GSDII using rAAV2 and rAAV1 vectors, with up to eight-fold over-expression of functional Gaa in the treated tissues (Fraites *et al.*, 2003; Fraites *et al.*, 2002; Mah *et al.*, 2004).

4.13 Cardiac Gene Transfer

Only recently has myocardium become a main target of rAAV-mediated gene transfer. Similar to intramuscular administration, a hurdle for efficient cardiac gene transfer is achieving widespread distribution of vector throughout the affected tissue. Most studies to date have implemented direct cardiac injection of vector, which have led to efficient transduction localized around the site of injection Champion *et al.*, 2003; Chu *et al.*, 2004; Li *et al.*, 2003; Yue *et al.*, 2003). Fraites *et al.* (2002) was able to demonstrate near-normal levels of cardiac GAA activity in a mouse model of GSDII via direct cardiac injection of a

rAAV2-based vector. Methods to further distribute vector transduction include induction of temporary cardiac ischemia followed by perfusion of vector, *ex vivo* infusion followed by transplantation, and the co-administration of vector with cardioplegic substances (Gregorevic *et al.*, 2004; Iwatate *et al.*, 2003).

4.14 GENE TRANSFER TO DIAPHRAGM

Due to its small size and thickness, the mouse diaphragm presents distinct challenges for the delivery of therapeutic agents. Previous diaphragm-targeted delivery methods have included via intravenous injection followed by transient occlusion of the vena cava and direct injection to the diaphragms of mice (Liu *et al.*, 2001; Petrof *et al.*, 1995; Stedman *et al.*, 1991; Yang *et al.*, 1998). Matrix-mediated vector delivery methods have been used extensively for gene therapy applications, particularly for non-viral gene delivery.

4.15 NEONATAL GENE TRANSFER

The therapeutic paradigm for most progressive diseases is that the younger the age at treatment, the higher the likelihood for therapeutic success. This is may be in part due to the minimal progression of disease phenotype and the potential to avoid immune response to the vector and/or transgene product. Several studies have examined the potential for treatment at early age timepoints with neonatal and even *in utero* gene therapy (Bouchard *et al.*, 2003). Rucker *et al.* (2004) demonstrated rAAV-mediated expression of GAA in a mouse model of GSDII after *in utero* administration. Although intraperitoneal delivery is effective in fetuses and neonates, its efficiency diminishes as the animals grow.

A recent study by Yue *et al.* demonstrated persistent cardiac transduction after direct injection of rAAV5 vectors into one-day-old mouse neonates (Yue *et al.*, 2003). Transduction events clustered mainly in the inner and outer myocardium, with some intermittent positively transduced cells in the middle layer. Several groups have also shown successful liver transduction after intravenous injection of rAAV vectors into neonatal mice (Daly *et al.*, 2001; Mah *et al.*, 2003). This example demonstrates that intravenous administration of alternate serotypes of rAAV vectors can achieve high levels of cardiac transduction.

5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – METHODS AND COMPOSITIONS FOR rAAV VECTOR DELIVERY TO DIAPHRAGM MUSCLE

The present example provides a safe, effective, and uniform method for delivery of recombinant adeno-associated virus vectors to the mouse diaphragm to facilitate gene therapy. The ability of rAAV serotypes 1, 2, and 5 to transduce the mouse diaphragm has been evaluated, and this example describes the application of a gel-based delivery method and demonstrates its utility for delivery of rAAV1, 2, and 5 to the mouse diaphragm. These results are the first to demonstrate efficient, uniform expression of a transgene in the murine diaphragm using rAAV vectors. Finally, the utility of this method was assessed using a mouse model (*Gaa*$^{-/-}$) of glycogen storage disease type II (GSDII) (Raben *et al.*, 1998), an autosomal recessive disorder that is characterized by respiratory insufficiency secondary to diaphragmatic weakness in affected juveniles (Moufarrej and Bertorini, 1993).

5.1.1 MATERIALS AND METHODS

5.1.1.1 PACKAGING AND PURIFICATION OF RECOMBINANT AAV1, 2, AND 5 VECTORS

The recombinant AAV2 plasmids pAAV-*lacZ* (Kessler *et al.*, 1996) and p43.2-*GAA* (Fraites *et al.*, 2002) have been described previously. Recombinant AAV vectors were generated, purified, and titered at the University of Florida Powell Gene Therapy Center Vector Core Lab as previously described (Zolotukhin *et al.*, 2002). Recombinant AAV particles based on serotypes 1, 2, and 5 were produced using pAAV-*lacZ,* whereas only rAAV1 particles (rAAV1-*GAA*) were packaged with p43.2-*GAA*.

5.1.1.2 VECTOR/VEHICLE PREPARATION

A sterile, bacteriostatic, water-soluble, glycerin-based gel was used as a vehicle for vector application to the diaphragm (K-Y® Sterile, Johnson & Johnson Medical, Arlington, TX). Individual doses of virus were diluted in sterile phosphate buffered saline (PBS) for a total volume of 10 μl and then added to 150 μl of gel in a 2 ml microcentrifuge tube. The virus-vehicle suspension was vortexed for one minute and then centrifuged for one minute at maximum speed. Free virus was diluted in sterile PBS for a total volume of 50 μl.

5.1.1.3 *IN VIVO* DELIVERY

All animal studies were performed in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee. Adult 129X1×C57BL/6 (wild type) or *Gaa*$^{-/-}$ mice (Raben *et al.*, 1998) were anesthetized using 2% isoflurane and restrained supine on a warmed operating surface. In a sterile field, after reaching a surgical plane of anesthesia, a midline incision was made through the skin extending from the xyphoid process to the suprapubic region. An incision was made through the abdominal wall following the *linea alba*. The abdominal walls were retracted laterally, the gall bladder was carefully separated from the rib cage, and the liver was carefully retracted from the diaphragm using sterile cotton swabs.

While lifting the xyphoid, free virus or virus mixed with vehicle were applied directly to the abdominal surface of the diaphragm. Free virus was applied using a pipet. To facilitate application of the gel to the diaphragm, a 22-gauge needle was used to puncture the bottom of the microcentrifuge tube and a plunger from a 3 cc syringe was used to force the gel through the hole and onto the diaphragm surface (FIG. 1). In some cases, a cotton-tipped applicator was used to ensure even spread over the entire diaphragm. After five min, the abdominal muscles were sutured and the skin was closed. Subcutaneous ampicillin (20-100 mg/kg) and buprenorphine (0.1 mg/kg) were administered prior to removing the animal from anesthesia.

5.1.1.4 ASSAYS OF β-GALACTOSIDASE AND GAA ENZYMATIC ACTIVITY

Six weeks after the surgical procedure and gene delivery, tissue lysates were assayed for enzyme activity using the Galacto-Star chemiluminescent reporter gene assay system (Tropix Inc., Bedford, MA). Protein concentrations for tissue lysates were determined using the Bio-Rad *DC* protein assay kit (Bio-Rad, Hercules, CA). For rAAV1-*GAA* treated

animals, enzymatic activity assays for GAA were performed six weeks after vector delivery as described previously (Fraites *et al.*, 2002). Tissue homogenates were assayed for GAA activity by measuring the cleavage of the synthetic substrate 4-methyl-umbelliferyl-α-D-glucoside (Sigma M9766, Sigma-Aldrich, St. Louis, MO) after incubation for 1 h at 37°C. Successful cleavage yielded a fluorescent product that emits at 448 nm, as measured with an FLx800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT). Protein concentration was measured as described above. Data are represented as nanomoles of substrate cleaved in one hour per milligram of total protein in the lysate (nmol/hr/mg).

5.1.1.5 Histological Assessment of Glycogen Clearance

Segments of treated and untreated diaphragm were fixed overnight in 2% glutaraldehyde in PBS, embedded in Epon, sectioned, and stained with periodic acid-Schiff (PAS) by standard methods (Raben *et al.*, 1998).

5.1.1.6 Biodistribution of Vector Genomes

Tissues were removed using sterile instruments and snap-frozen in liquid nitrogen. Total cellular DNA was extracted from tissue homogenates using a Qiagen DNeasy® kit per the manufacturer's instructions (Qiagen, Valencia, CA). Nested PCR™ reactions were performed as follows: 1.5 μg total DNA was used as a template for the initial PCR™ amplification using the sense primer 5'-AGCTGGCGTAATAGCGAAGA-3' (SEQ. ID NO:1) and reverse primer 5'-CGCGTCTCTCCAGGTAGCGAA-3' (SEQ. ID NO:2), yielding a 1486-bp product. The PCR™ product was purified using the Qiagen MinElute PCR™ purification kit per the manufacturer's instructions, followed by PCR™ amplification using the sense primer 5'-CGGTGATGGTGCTGCGTTGGAG-3' (SEQ. ID NO:3) and reverse primer 5'-TCGACGTTCAGACGTAGTGT-3' (SEQ. ID NO:4), resulting in a final product of 333 bp. All reactions were performed under the following conditions: hot start denaturation at 94°C for five min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 2 min. Products were electrophoresed and analyzed using a 2% agarose gel.

5.1.2 RESULTS

5.1.2.1 EFFICIENCY OF TRANSDUCTION USING GEL-BASED DELIVERY OF RAAV *IN VIVO*

The efficiency of rAAV delivery using the gel-based method was compared to free virus delivery using β-galactosidase as a reporter gene (FIG. 2A). Direct particle-to-particle comparisons of histochemistry from free-virus-treated animals (left column) versus gel-based delivery (right column) indicate an increased efficiency of transduction for all serotypes using the latter method. Quantitative analysis of tissue lysates from these animals using the Galacto-Star enzymatic assay for β-galactosidase confirms these results (FIG. 2B). Activities for subjects treated with gel-vector suspensions had higher activities for all three serotypes.

5.1.2.2 VARYING TROPISMS OF RAAV SEROTYPES 1, 2, AND 5 FOR DIAPHRAGM MUSCLE *IN VIVO*

The results from FIG. 2A and FIG. 2B also indicate a distinct gradient of tropism for mouse diaphragm among the three tested serotypes. Qualitatively, rAAV1 vectors led to the most intense staining under both the free virus and gel-based conditions. Differences between rAAV2 and rAAV5 were hard to distinguish in the free virus case due to the low levels of transduction for both vectors, but the gel-mediated subjects demonstrated a clear preference for rAAV2 compared to rAAV5. These results are further verified in FIG. 2B, which indicates higher levels of enzyme activity for rAAV2 gel suspensions compared to rAAV5. Taken together, the results of histochemical staining and enzymatic activity indicate: (1) a substantial increase in viral transduction using a physical delivery system; and (2) a clearly enhanced mouse diaphragm tropism for rAAV1, and a potentially important difference between rAAV2 and rAAV5.

5.1.2.3 GEL-BASED DELIVERY OF RAAV1-*GAA* RESULTS IN BIOCHEMICAL CORRECTION OF DIAPHRAGMS IN *GAA*$^{-/-}$ MICE.

Having demonstrated increased transduction of the mouse diaphragm using the gel-based method, the ability of this method to restore enzymatic activity in a mouse model of glycogen storage disease type II (GSDII; MIM 232300), a lysosomal glycogen storage disease caused by a lack or deficiency of the lysosomal enzyme, acid α-glucosidase (GAA; EC 3.2.1.20) was assessed. The mouse model of this disease stores glycogen in all tissues, with significant pathologies in the heart and skeletal muscle (Raben *et al.*, 1998). The use of rAAV vectors to restore enzymatic and functional activity in skeletal and cardiac muscle in

these mice was previously characterized (Fraites *et al.*, 2002). Coupled with new findings using a gel-based delivery method, it was hypothesized that gel-based delivery of rAAV1-GAA would be able to restore GAA activity in *Gaa*$^{-/-}$ diaphragms and, in turn, reverse lysosomal glycogen accumulation.

Using rAAV1-*GAA* vectors, increases in diaphragmatic transduction in *Gaa*$^{-/-}$ mice similar to those seen in control mice with β-galactosidase vector were found. GAA enzymatic activities were restored to 50% of wild type with free vector, and were further increased to 120% of normal levels using a vector-gel suspension (FIG. 3A). These activities had a profound effect in glycogen storage, as assessed by periodic acid-Schiff's reagent (PAS) staining (FIG. 3B). Dark pink vacuoles, indicative of stored glycogen, are observed in free-vector-treated diaphragms from *Gaa*$^{-/-}$ mice whereas a near-complete reversal of glycogen accumulation from diaphragms is seen in gel-treated mice.

5.1.2.4 Biodistribution of rAAV Genomes After Gel-Based Delivery

Since a secondary advantage of physical delivery systems may be the ability to restrict viral spread, it was also sought to determine which tissues endocytosed the viral vectors after gel-based delivery. To this end, various tissues from rAAV1-βgal gel-treated mice were harvested and total cellular DNA was extracted. Using a nested PCR™ technique, a portion of the β-galactosidase gene was amplified from vector genomes (FIG. 4). As expected, vector genomes could be detected in treated diaphragms. Vector genomes could not be detected in any other tissue examined (including sections of the peritoneal wall and liver adjacent to the diaphragm); however, it is possible that more sensitive detection methods (such as real-time PCR™) would detect trace amounts of vector genomes.

5.1.3 Discussion

Transduction events for recombinant adeno-associated viruses can be separated into five general stages: (1) binding and entry (endocytosis); (2) endosomal processing and escape; (3) transcytosis; (4) nuclear import and uncoating; and (5) genome conversion, including second-strand synthesis (or alternatively self-complementation), followed by genome concatemerization and/or integration into the host chromosome. This example describes, for the first time, an improvement in the process whereby enhancement of the first step of this process using a physical method prolongs viral dwell time and increases the

efficiency of transduction by providing longer viral particle exposure times to receptors on target tissues.

Carrier molecules and delivery agents have been used extensively for gene therapy applications, particularly for non-viral gene delivery. With regard to viral vectors, recombinant adenoviruses have been used in concert with a variety of agents in order to increase or prolong bioavailability, thereby enhancing the efficiency of delivery. March *et al.* (1995) reported the use of poloxamer 407, a hydrogel which exhibits potentially useful, thermo-reversible gelation, enabling formulation at low temperature with subsequent hardening to a robust gel at room and physiologic temperatures. They demonstrated increased transduction of vascular smooth muscle cells *in vitro*, with similar findings reported *in vivo* by Van Belle *et al.* (1998). Unfortunately, poloxamers have recently been shown to have adverse effects on adeno-associated virus stability (Croyle *et al.*, 2001). Likewise, thixotropic solutions have also shown promise for enhancing adenovirus-mediated transduction of airway epithelia (Seiler *et al.*, 2002). Several other promising agents have also been effectively used with adenovirus vectors, including β-cyclodextrins, surfactants, and collagen- or gelatin-based matrices.

While extensive testing of potential adenovirus formulations has been reported, few similar studies are extant for adeno-associated viruses. Most of the available literature describes formulations that increase stability for storage or purification, but few reports address the need for augmented physical delivery of viral particles *in vivo*. These inventors and collaborators have previously described the use of microsphere-conjugated rAAV for systemic delivery of viral vectors, in which it was possible to significantly increase the transduction efficiency in target tissue beds *in vivo* by increasing vector dwell time (Mah *et al.*, 2002). Similarly, a number of groups are currently developing capsid-modified rAAV vectors to target specific vascular beds upon systemic delivery. To date, however, the literature is devoid of other examples of physical delivery agents or methods to improve rAAV delivery to tissue surfaces, such as skin, blood vessel adventitia, or diaphragm.

The methods described herein for diaphragmatic delivery of rAAV vector-based composition reliy on retention of vector on the peritoneal surface of the diaphragm. Local delivery using this strategy is clinically achievable by endoscopic delivery and has the added benefit of reduced risk associated with systemic vascular delivery. While this method has been specifically applied to the murine diaphragm, the inventors believe that such gel-based AAV compositions have broad utility for improving the transduction efficiency of the

vectors in a variety of tissues. (One important use contemplated by the inventors is that of topical application of gel-based rAAV compositions formulated for wound or burn healing).

Comparisons of rAAV serotype tropisms for skeletal muscle have already been reported (Fraites *et al.*, 2002; Chao *et al.*, 2001; Chao *et al.*, 2000; Hauck and Xiao, 2003). Several recombinant AAV vectors based on alternative serotypes have demonstrated greater transduction efficiencies in skeletal muscle than serotype 2. In particular, several reports have shown nearly one log greater expression of a variety of transgenes when packaged in rAAV1 capsids compared to rAAV2. Similar findings have been reported with rAAV6, although this serotype has not been as widely studied (Hauck and Xiao, 2003; Moufarrej and Bertorini, 1993). Clear differences in serotype tropism were observed between rAAV1 and the other two serotypes in the context of gel-based delivery and free virus administration, with significant differences observed between rAAV1 and rAAV5 ($p<0.1$). The eight-fold over-expression of GAA in Gaa-deficient diaphragms after delivery of free rAAV1-*GAA* compared to serotype 2 (FIG. 2B, AAV1 Free *vs.* AAV2 Free) is nearly identical to prior observations after direct intramuscular administration of the same two vectors in *tibialis anterior* muscles of $Gaa^{-/-}$ mice (Fraites *et al.*, 2002), indicating a conserved rAAV1 tropism for skeletal muscle.

5.2 EXAMPLE 2 – MURINE MODELS OF GLYCOGEN STORAGE DISEASE TYPE II

For these studies, two different mouse models of GSDII are employed. For the gene therapy studies, a knockout mouse model of GSDII ($Gaa^{-/-}$) developed by Raben *et al.* is used. This mouse model was generated by the insertion of a neomycin gene cassette into exon 6 of the murine *Gaa* gene and recapitulates the human disease in that there is progressive skeletal muscle weakening and glycogen storage (Raben *et al.*, 1998).

An alternative mouse model of GSDII (Mck-T-$GAA/Gaa^{-/-}$) in which human GAA can be conditionally-expressed in skeletal muscle in response to tetracycline in the context of the $Gaa^{-/-}$ background is also used (Gossen and Bujard, 1992; Raben *et al.*, 2001). GAA expression can be completely shut off when the animals are fed doxycycline (a tetracycline derivative)-supplemented food (FIG. 5). Raben *et al.* (2002) showed that glycogen clearance in Mck-T-$GAA/Gaa^{-/-}$mice could be achieved with modest levels of cardiac GAA expression in young animals, whereas, in older adult animals, supraphysiologic levels lead to only 40-50% glycogen clearance. Conversely, in skeletal muscle, greater than 8-fold normal levels of GAA activity were required to achieve complete clearance of glycogen in

young animals. Using this conditionally-expressing model, the relationship of the severity of disease phenotype, or the stage of disease progression, may be characterized with the propensity for biochemical and functional correction. While clearance of glycogen is a crucial aspect in the successful treatment of GSDII, it is possible that a reduction, rather than complete clearance, of glycogen in affected tissues may result in significantly improved muscle function.

5.2.1 Recombinant AAV-Mediated Transduction in Adult Mouse Heart and Diaphragm

A study in which either rAAV1 or rAAV2 vector was administered via direct cardiac injection to adult *Gaa*$^{-/-}$ mice resulted in near-normal levels of cardiac GAA activity with both serotypes (FIG. 6). Interestingly, when administered intravenously in neonate mice, there was a dramatic difference in cardiac GAA levels between the two serotypes. These results are further discussed below.

As the diaphragm is severely affected in GSDII and many other muscular dystrophies, the inventors sought to develop a new method of rAAV vector delivery to enhance diaphragm transduction. A gel biopolymer formulation was used to deliver 1×10^{11} particles of rAAV-CMV-*lacZ* to adult 129X1xC57BL/6 mouse diaphragms. As shown in FIG. 2A, gross histochemical comparison of *lacZ* expression indicates an increased efficiency of transduction for all rAAV serotypes delivered in the gel. Quantitative enzyme detection analysis further confirmed this observation. Furthermore, it was also observed that rAAV serotype 1 vectors transduced diaphragm more efficiently than rAAV2- and 5-based vectors, whether delivered free or in gel vehicle.

The potential utility of matrix-mediated delivery of rAAV in a mouse model of GSDII was also investigated. 1×10^{11} particles of therapeutic rAAV1 encoding the CMV promoter driven-human *GAA* gene (rAAV1-CMV-*GAA*) was administered directly to the diaphragm either in free or gel-based formulations. GAA enzymatic activities were restored to 50% of wild-type with free vector, and were further increased to 120% of normal levels using the vector-gel suspension. Furthermore, the high levels of GAA expression had a profound effect on glycogen storage, as assessed by periodic acid-Schiff's (PAS) reagent staining. As shown in FIG. 3B, stored glycogen (indicated by dark-stained vacuoles) is observed in free vector treated diaphragms, whereas a substantial reversal of glycogen accumulation is seen in diaphragms of gel-treated mice. In sum, these studies demonstrate

the use of matrix-mediated delivery of rAAV vector to diaphragm for the treatment of skeletal myopathies.

5.2.2 Recombinant AAV-Mediated Transduction in Mouse Neonates

Although diaphragmatic transduction posed a technical challenge in adult mouse models, transduction of mouse neonate diaphragms has been much simpler. In initial studies, simple intraperitoneal injection of 1×10^{11} particles rAAV2-CMV-*lacZ* vector at one-day of age resulted in almost complete transduction of the diaphragm, as assessed by X-gal staining four weeks post-injection.

As described above, direct cardiac administration of rAAV1 and rAAV2 vectors to adult animals leads to normal levels of cardiac GAA activity. Studies were performed in which rAAV2 vectors encoding for CMV-*GAA* were administered intravenously to one-day-old $Gaa^{-/-}$ mice. Similar to the adult animal studies, intravenous administration of rAAV2 to neonates also resulted in near-normal levels of cardiac GAA activity. Conversely, intravenous administration of 5×10^{10} particles of an rAAV1 vector in neonates resulted in supraphysiologic levels of GAA expression in the hearts of treated animals, with an average of 650% of normal levels, eleven months post-injection. In addition, levels of diaphragm, lung, and quadriceps GAA enzyme activity levels were above the therapeutic threshold of 20% (FIG. 7). As shown in FIG. 8, almost complete clearance of stored glycogen was observed in the hearts of treated animals, as determined by PAS staining of heart sections. Biodistribution analysis of vector genomes in treated animals suggested that the high levels of heart and diaphragm GAA activity were a result of rAAV-mediated transduction of those tissues, with studies suggesting approximately 0.42 vector genomes per diploid cell in transduced heart. Despite high levels of GAA activity in the lung, no significant transduction of the lung was noted, as determined by extremely low vector genome copies and a lack of transgene specific RT-PCR product, suggesting that the heart is secreting expressed enzyme, which in turn is taken up by the lung. Access to the bloodstream and the ability to promote systemic circulation of secreted proteins makes the heart as a depot organ, in which therapeutic enzyme can be produced and secreted, an interesting concept. As shown in FIG. 9, a marked improvement was noted in soleus muscle function in treated mice as compared to age-matched control animals and even animals five months younger in age. Although significant functional improvement was noted in soleus muscle, the levels of GAA activity were not above the 20% therapeutic threshold. Several potential explanations

include that only minimal levels of GAA activity are required for functional correction of the soleus muscle, the soleus had substantial GAA activity at an earlier timepoint protecting it from severe muscle pathology, or the improved heart and diaphragm function resulted in overall better circulation and general health which was to some extent protective. These results demonstrate the use of intravenous administration of alternate rAAV serotype vectors to transduce multiple target tissues.

Recent data suggests that other serotypes may be even more efficient at globally transducing skeletal and cardiac muscle than rAAV1 vectors. Another AAV serotype, serotype 9, has been developed as a gene therapy vector (Limberis *et al.*, 2004; Wang *et al.*, 2004) . As shown in FIG. 10, direct cardiac administration of rAAV1 or rAAV9 vectors encoding for CMV-*lacZ* to neonatal mice resulted in substantially higher levels of transgene expression from the rAAV9 vector than the rAAV1 vector, four-weeks post-injection. These results suggest that rAAV9-based vectors may be more effective vectors for cardiac-targeted gene transfer.

5.2.3 Gene Expression Profiling of GSDII

The potential for modifying genes to be involved in the pathology of GSDII has been proposed, though to-date, none have been clearly identified. Studies have been performed in an attempt to identify such modifying genes. GAA-deficient myoblasts isolated from *Gaa*$^{-/-}$ mice or from GSDII patient samples were transduced with rAAV1-CMV-*GAA* or control vector. RNA was isolated from the cells and processed and analyzed on Affymetrix Murine Genome U74Av2 or Human Genome U133A Plus 2.0 GeneChips. The geometric mean hybridization intensities were analyzed to identify genes that differentiated among the three treatment classes: mock infection, rAAV1-*factor VIII* (*FVIII*) infection (control), and rAAV1-*GAA* infection. Of the 7676 genes considered in the murine myoblast analysis, 53 genes differentiated among the treatment classes and could function as classifiers of treatment response ($P \leq 0.001$). Of the 53 identifying genes, five genes were specifically upregulated in response to rAAV1-*hGAA* infection. In the human myoblast study, 10 different genes were identified to be up- or down-regulated in response to the specific gain in GAA activity. As a control, the *FVIII* gene was identified in those samples that were infected with control rAAV1-*FVIII* with a p-value < 0.0001. The GAA gene was not identified, as a 3' UTR truncated form of the GAA gene was used, the deleted regions of which the Affymetrix probe sets were targeted against. On the outset, two identified genes

in particular stood out as potential candidates for further investigation. The differential expression of the candidate genes was confirmed by RT-PCR. The first candidate, stomatin, is a membrane protein shown to be associated with late endosomes/lysosomes. Overexpression of stomatin has been shown to inhibit GLUT-1 glucose transporter activity. The second candidate, laforin interacting protein 1, has phosphatase and carbohydrate binding sites and is associated with laforin. A lack of laforin is associated with Lafora disease, a disorder or glycogen metabolism.

5.2.4 VECTOR BIODISTRIBUTION AND TRANSGENE EXPRESSION OF ALTERNATE SEROTYPE RECOMBINANT ADENO-ASSOCIATED VIRUS VECTORS

Vector is administered intravenously or *via* intracardiac injection to one-day-old neonate or eight-week-old *Gaa*$^{-/-}$ mice. Furthermore, vector is administered directly to diaphragm in adult mice only. Intraperitoneal injection in neonate mice resulted in significant diaphragmatic transduction.

5.2.4.1 VECTOR PRODUCTION

Packaging of rAAV serotypes 1, 2, 5, 6, 8, and 9 vectors is performed using the traditional transfection method used for AAV2 vector production described by Zolotukhin *et al* (1999 and 2002) . Helper plasmids that retain the AAV2 *rep* gene and alternate AAV serotype *cap* genes may be used. Since helper plasmids still retain the AAV2 *rep* gene, all AAV plasmid constructs using AAV2 inverted terminal repeats (ITRs) are packageable with the new helper plasmids. Recombinant virus may be purified by conventional means, including for example, an iodixanol density gradient ultracentrifugation followed by anion exchange chromatography. Vector preparation purity may also be assessed by conventional means, including for example SDS-PAGE followed by silver staining to visualize protein content. Vector genome titers may be determined by conventional means, including for example, dot-blot hybridization.

5.2.4.2 ADMINISTRATION OF VECTOR TO MOUSE NEONATES

One-day-old neonate mice are anesthetized by induction of hypothermia. A 29½G tuberculin syringe is used to deliver 1×10^{13} vector genomes/kg *via* the superficial temporal vein or directly into the heart (at a max. volume of 30 μl for intravenous injection or 10 μl for intracardiac injection), both of which are easily visualized during the first two days post-

birth. An *n* of at least 5 animals are used for each serotype and route of administration (6 serotypes × 2 routes × 5 = 60 animals). Any bleeding that results from the injection may be controlled by applying light pressure to the injection site using a sterilized cotton swab until bleeding stops. Treated animals are then returned to the mothers, and normal housing and care.

5.2.4.3 Administration of Vector to Adult Mice

Eight-week-old animals are anesthetized with 2% inhaled isoflurane. As with the neonate study, an *n*=5 is used for each serotype/route of administration for a total of 60 animals. After reaching a surgical plane of anesthesia, an incision is made through the skin to expose the external jugular vein. Intravenous injections of 1×10^{13} vector genomes/kg are made using a 29½G tuberculin syringe. Direct pressure to the injection site is applied using a sterile cotton swab until bleeding is stopped. The incisions are sutured closed. For direct injection into the cardiac muscle, a 22G catheter connected to a SAR-830AP rodent ventilator (CWE, Ardmore, PA) may be used to intubate anesthetized animals and facilitate ventilation. A left thoracotomy may be performed to expose the left ventricle. Vector is directly injected using a 29½G tuberculin syringe and the incision is then sutured closed.

5.2.4.4 Matrix-Mediated Delivery of rAAV to Murine Diaphragm

For direct diaphragm delivery studies, an *n*=5 animals per serotype is used for a total of 30 mice. Individual doses (1×10^{13} vector genomes/kg) of virus is diluted in sterile phosphate buffered saline (PBS) for a total volume of 10 μL and then added to biopolymer to a final volume of 150 μL. Eight-week-old *Gaa*$^{-/-}$ mice are anesthetized using 2% inhaled isoflurane and restrained supine on a warmed operating surface. After reaching a surgical plane of anesthesia, a midline incision is made, the abdominal walls retracted laterally, the gall bladder separated from the rib cage, and the liver carefully retracted from the diaphragm. While lifting the xyphoid, vector-matrix mixtures are applied directly to the abdominal surface of the diaphragm (Mah *et al.*, 2004). The incision is sutured closed.

5.2.4.5 Tissue Analysis

Four weeks after vector administration, the diaphragm, liver, spleen, kidney, lung, heart, soleus, quadriceps, tibialis anterior, gastrocnemius and gonads are isolated and

divided for the following assays: (1) *lacZ* enzyme detection assay, (2) detection of viral genomes, and (3) histopathological examination, as described below.

5.2.4.6 DETECTION OF TRANSGENE β-GALACTOSIDASE EXPRESSION

Histochemical staining with X-gal is performed as described previously (Kessler *et al.*, 1996). Tissue is fixed in ice-cold 2% formaldehyde, 0.2% glutaraldehyde, rinsed in sterile PBS, then stained in X-gal stain solution (1 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS) overnight at room temperature, protected from light. Cells expressing *lacZ* are stained blue.

Detection of β-galactosidase enzyme is performed on crude homogenates of tissue using the Galacto-*Star*™ chemiluminescent reporter gene assay system (Tropix Inc., Bedford, MA) per the manufacturer's instructions. Protein concentrations for tissue lysates may be determined using the Bio-Rad *DC* protein assay kit (Bio-Rad, Hercules, CA). Enzyme activities are reported as relative light units (RLU) per μg protein.

5.2.4.7 ASSESSMENT OF *IN VIVO* BIODISTRIBUTION OF VECTOR GENOMES

Detection of rAAV vector genomes is assessed as described previously Mingozzi *et al.*, 2002). Total cellular DNA is extracted from tissues using the DNeasy kit (QIAGEN, Valencia, CA) per the kit protocols. Co-amplification of the *lacZ* gene (found in the vector genome) and the endogenous murine hypoxanthine guanine phosphoribosyl transferse (*Hprt*) gene are performed by polymerase chain reaction (PCR) using biotinylated primers on 1.5 μg total DNA as a template. Primer pairs for *lacZ* (5'-CGGTGATGGTGCTGCGTT GGAG-3' (SEQ ID NO:1) and 5'-TCGACGTTCAGACGTAGTGT-3') (SEQ ID NO:2) and *Hprt* (5'-GCTGGTGAAAAGGACCTCT-3' (SEQ ID NO:3) and 5'-CACAGGACTAGAA CACCTGC-3' (SEQ ID NO:4)), result in final PCR products of 333 bp and 1.1 kb, respectively. In addition, standard controls include 0 (negative control), 0.01, 0.05, 0.1, 0.5 and 1 pg linearized CMV-*lacZ* plasmid DNA spiked into 1.5 μg control cellular DNA isolated from untreated mouse tissue. All reactions are performed under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. Products are electrophoresed on a 2% agarose gel followed by transfer to a nylon membrane and visualized using the Southern-*Star* system (Applied Biosystems, Bedford, MA) as per the kit

protocol. Densitometric analysis of resulting bands is performed using *Scion Image* Release Beta 4.0.2 software (Scion Corporation, Frederick, Maryland) and ratios of *lacZ/Hprt* band intensity are calculated. Gene copy numbers are estimated from the standard curve generated from the standard controls.

5.2.4.8 HISTOPATHOLOGICAL EXAMINATION OF TISSUES

After necropsy, isolated tissue sections are fixed in 10% neutral buffered formalin and processed for paraffin embedding by standard techniques. Tissue sections (5 μm thickness) are stained with hematoxylin and eosin (H&E) using standard methods.

5.3 EXEMPLARY HUMAN THERAPEUTIC AGENTS USEFUL IN THE PRACTICE OF THE PRESENT INVENTION

As an example of the therapeutic agents that may be delivered to mammalian muscle and cardiac tissues, the following DNA and protein sequences are included as illustrative embodiments of the present method. For the treatment of certain muscular dystrophies, expression of the human DMD gene in selected muscle tissues is preferred to ameliorate the defect and provide biologically-effective amounts of the protein to selected cells. Likewise, for Pompe's Disease, a deficiency in acid α-glucosidase (GAA), an illustrative use of the disclosed methods and composition employs a mammalian GAA gene, such as the human GAA gene identified in GenBank to express biologically- and therapeutically-effective amounts of the polypeptide in selected cells. These are but two examples of human therapeutic genes which are contemplated to find utility in the practice of the present invention.

5.3.1 HUMAN GAA GENE

Human GAA Gene Sequence REF: GenBank NM_000152) (SEQ ID NO:5)

GCGCCTGCGCGGGAGGCCGCGTCACGTGACCCACCGCGGCCCCGCCCCGCGACGAGCTCCCGCCGGTCACGTGA
CCCGCCTCTGCGCGCCCCCGGGCACGACCCCGGAGTCTCCGCGGGCGGCCAGGGCGCGCGTGCGCGGAGGTGAG
CCGGGCCGGGGCTGCGGGGCTTCCCTGAGCGCGGGCCGGGTCGGTGGGGCGGTCGGCTGCCCGCGCCGGCCTCT
CAGTTGGGAAAGCTGAGGTTGTCGCCGGGGCCGCGGGTGGAGGTCGGGGATGAGGCAGCAGGTAGGACAGTGAC
CTCGGTGACGCGAAGGACCCCGGCCACCTCTAGGTTCTCCTCGTCCGCCCGTTGTTCAGCGAGGGAGGCTCTGG
GCCTGCCGCAGCTGACGGGGAAACTGAGGCACGGAGCGGGCCTGTAGGAGCTGTCCAGGCCATCTCCAACCATG
GGAGTGAGGCACCCGCCCTGCTCCCACCGGCTCCTGGCCGTCTGCGCCCTCGTGTCCTTGGCAACCGCTGCACT
CCTGGGGCACATCCTACTCCATGATTTCCTGCTGGTTCCCCGAGAGCTGAGTGGCTCCTCCCCAGTCCTGGAGG

AGACTCACCCAGCTCACCAGCAGGGAGCCAGCAGACCAGGGCCCCGGGATGCCCAGGCACACCCCGGCCGTCCC
AGAGCAGTGCCCACACAGTGCGACGTCCCCCCAACAGCCGCTTCGATTGCGCCCCTGACAAGGCCATCACCCA
GGAACAGTGCGAGGCCCGCGGCTGCTGCTACATCCCTGCAAAGCAGGGGCTGCAGGGAGCCCAGATGGGGCAGC
CCTGGTGCTTCTTCCCACCCAGCTACCCCAGCTACAAGCTGGAGAACCTGAGCTCCTCTGAAATGGGCTACACG
GCCACCCTGACCCGTACCACCCCCACCTTCTTCCCCAAGGACATCCTGACCCTGCGGCTGGACGTGATGATGGA
GACTGAGAACCGCCTCCACTTCACGATCAAAGATCCAGCTAACAGGCGCTACGAGGTGCCCTTGGAGACCCCGC
GTGTCCACAGCCGGGCACCGTCCCCACTCTACAGCGTGGAGTTCTCCGAGGAGCCCTTCGGGGTGATCGTGCAC
CGGCAGCTGGACGGCCGCGTGCTGCTGAACACGACGGTGGCGCCCCTGTTCTTTGCGGACCAGTTCCTTCAGCT
GTCCACCTCGCTGCCCTCGCAGTATATCACAGGCCTCGCCGAGCACCTCAGTCCCCTGATGCTCAGCACCAGCT
GGACCAGGATCACCCTGTGGAACCGGGACCTTGCGCCCACGCCCGGTGCGAACCTCTACGGGTCTCACCCTTTC
TACCTGGCGCTGGAGGACGGCGGGTCGGCACACGGGGTGTTCCTGCTAAACAGCAATGCCATGGATGTGGTCCT
GCAGCCGAGCCCTGCCCTTAGCTGGAGGTCGACAGGTGGGATCCTGGATGTCTACATCTTCCTGGGCCCAGAGC
CCAAGAGCGTGGTGCAGCAGTACCTGGACGTTGTGGATACCCGTTCATGCCGCCATACTGGGGCCTGGGCTTC
CACCTGTGCCGCTGGGGCTACTCCTCCACCGCTATCACCCGCCAGGTGGTGGAGAACATGACCAGGGCCCACTT
CCCCCTGGACGTCCAATGGAACGACCTGGACTACATGGACTCCCGGAGGGACTTCACGTTCAACAAGGATGGCT
TCCGGGACTTCCCGGCCATGGTGCAGGAGCTGCACCAGGGCGGCCGGCGCTACATGATGATCGTGGATCCTGCC
ATCAGCAGCTCGGGCCCTGCCGGGAGCTACAGGCCCTACGACGAGGGTCTGCGGAGGGGGGTTTTCATCACCAA
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CCCTGGCCTGGTGGAGGACATGGTGGCTGAGTTCCATGACCAGGTGCCCTTCGACGGCATGTGGATTGACATG
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ACAACCTGCACAACCTCTACGGCCTGACCGAAGCCATCGCCTCCCACAGGGCGCTGGTGAAGGCTCGGGGGACA
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GGGCCGACGTCTGCGGCTTCCTGGGCAACACCTCAGAGGAGCTGTGTGTGCGCTGGACCCAGCTGGGGGCCTTC
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CACCAGCTCCTGTGGGGGGAGGCCCTGCTCATCACCCCAGTGCTCCAGGCCGGGAAGGCCGAAGTGACTGGCTA
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CAGCTCCCCGTGAGCCAGCCATCCACAGCGAGGGGCAGTGGGTGACGCTGCCGGCCCCCCTGGACACCATCAAC
GTCCACCTCCGGGCTGGGTACATCATCCCCCTGCAGGGCCCTGGCCTCACAACCACAGAGTCCCGCCAGCAGCC
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GCAGGTCCTCTCCAACGGTGTCCCTGTCTCCAACTTCACCTACAGCCCCGACACCAAGGTCCTGGACATCTGTG
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GAGGCTGGTTCCCCAGGGAAGCAGAGCCTGTGTGCGGGCAGCAGCTGTGTGCGGGCCTGGGGGTTGCATGTGTC
ACCTGGAGCTGGGCACTAACCATTCCAAGCCGCCGCATCGCTTGTTTCCACCTCCTGGGCCGGGGCTCTGGCCC
CCAACGTGTCTAGGAGAGCTTTCTCCCTAGATCGCACTGTGGGCCGGGGCCTGGAGGGCTGCTCTGTGTTAATA

AGATTGTAAGGTTTGCCCTCCTCACCTGTTGCCGGCATGCGGGTAGTATTAGCCACCCCCCTCCATCTGTTCCC
AGCACCGGAGAAGGGGGTGCTCAGGTGGAGGTGTGGGGTATGCACCTGAGCTCCTGCTTCGCGCCTGCTGCTCT
GCCCCAACGCGACCGCTTCCCGGCTGCCCAGAGGGCTGGATGCCTGCCGGTCCCCGAGCAAGCCTGGGAACTCA
GGAAAATTCACAGGACTTGGGAGATTCTAAATCTTAAGTGCAATTATTTTAATAAAAGGGGCATTTGGAATC

5.3.2 HUMAN GAA PROTEIN

Human GAA Protein –REF: GenBank NM_000152 (SEQ ID NO:6)

MGVRHPPCSHRLLAVCALVSLATAALLGHILLHDFLLVPRELSGSSPVLEETHPAHQQGASRPGPRDA
QAHPGRPRAVPTQCDVPPNSRFDCAPDKAITQEQCEARGCCYIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLS
SSEMGYTATLTRTTPTFFPKDILTLRLDVMMETENRLHFTIKDPANRRYEVPLETPRVHSRAPSPLYSVEFSEE
PFGVIVHRQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTRITLWNRDLAPTPGAN
LYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRSTGGILDVYIFLGPEPKSVVQQYLDVVGYPFMP
PYWGLGFHLCRWGYSSTAITRQVVENMTRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRY
MMIVDPAISSSGPAGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEFHDQVPF
DGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQFLSTHYNLHNLYGLTEAIASHRA
LVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWEQLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVR
WTQLGAFYPFMRNHNSLLSLPQEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKD
SSTWTVDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPIEALGSLPPPPAAPREPAIHSEGQWVTLP
APLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVALTKGGEARGELFWDDGESLEVLERGAYTQVIFLARN
NTIVNELVRVTSEGAGLQLQKVTVLGVATAPQQVLSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC

5.3.3 HUMAN DMD DYSTROPHIN GENE (DUCHENNE BECKER TYPE)

Human DMD (Dystrophin) Gene Sequence REF: GenBank M18533 (SEQ ID NO:7)

TTTTCAAAATGCTTTGGTGGGAAGAAGTAGAGGACTGTTATGAAAGAGAAGATGTTCAAAAGAAAACATTCACA
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AAATGTAATGAAAAATATCATGGCTGGATTGCAACAAACCAACAGTGAAAAGATTCTCCTGAGCTGGGTCCGAC
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ATTGAAGCCATCCAGGAAGTGGAAATGTTGCCAAGGCCACCTAAAGTGACTAAAGAAGAACATTTTCAGTTACA
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GTCAGCCTATGCTGCTCCGAGTGGTTGGCAGTCAAACTTCGGACTCCATGGGTGAGGAAGATCTTCTCAGTCCT
CCCCAGGACACAAGCACAGGGTTAGAGGAGGTGATGGAGCAACTCAACAACTCCTTCCCTAGTTCAAGAGGAAG
AAATACCCCTGGAAAGCCAATGAGAGAGGACACAATGTAG

5.3.4 HUMAN DMD DYSTROPHIN POLYPEPTIDE (DUCHENNE BECKER TYPE)

Human DMD (Dystrophin) Protein Sequence REF: GenBank M18533 (SEQ ID NO:8)

MLWWEEVEDCYEREDVQKKTFTKWVNAQFSKFGKQHIENLFSDLQDGRRLLDLLEGLTGQKLPKEKGSTRVHAL
NNVNKALRVLQNNNVDLVNIGSTDIVDGNHKLTLGLIWNIILHWQVKNVMKNIMAGLQQTNSEKILLSWVRQST
RNYPQVNVINFTTSWSDGLALNALIHSHRPDLFDWNSVVCQQSATQRLEHAFNIARYQLGIEKLLDPEDVDTTY
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KSYAYTQAAYVTTSDPTRSPFPSQHLEAPEDKSFGSSLMESEVNLDRYQTALEEVLSWLLSAEDTLQAQGEISN
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QAVTTTQPSLTQTTVMETVTTVTTREQILVKHAQEELPPPPPQKKRQITVDSEIRKRLDVDITELHSWITRSEA
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LGAKVTERKQQLEKCLKLSRKMRKEMNVLTEWLAATDMELTKRSAVEGMPSNLDSEVAWGKATQKEIEKQKVHL
KSITEVGEALKTVLGKKETLVEDKLSLLNSNWIAVTSRAEEWLNLLLEYQKHMETFDQNVDHITKWIIQADTLL
DESEKKKPQQKEDVLKRLKAELNDIRPKVDSTRDQAANLMANRGDHCRKLVEPQISELNHRFAAISHRIKTGKA
SIPLKELEQFNSDIQKLLEPLEAEIQQGVNLKEEDFNKDMNEDNEGTVKELLQRGDNLQQRITDERKREEIKIK
QQLLQTKHNALKDLRSQRRKKALEISHQWYQYKRQADDLLKCLDDIEKKLASLPEPRDERKIKEIDRELQKKKE
ELNAVRRQAEGLSEDGAAMAVEPTQIQLSKRWREIESKFAQFRRLNFAQIHTVREETMMVMTEDMPLEISYVPS
TYLTEITHVSQALLEVEQLLNAPDLCAKDFEDLFKQEESLKNIKDSLQQSSGRIDIIHSKKTAALQSATPVERV
KLQEALSQLDFQWEKVNKMYKDRQGRFDRSVEKWRRFHYDIKIFNQWLTEAEQFLRKTQIPENWEHAKYKWYLK
ELQDGIGQRQTVVRTLNATGEEIIQQSSKTDASILQEKLGSLNLRWQEVCKQLSDRKKRLEEQKNILSEFQRDL
NEFVLWLEEADNIASIPLEPGKEQQLKEKLEQVKLLVEELPLRQGILKQLNETGGPVLVSAPISPEEQDKLENK
LKQTNLQWIKVSRALPEKQGEIEAQIKDLGQLEKKLEDLEEQLNHLLLWLSPIRNQLEIYNQPNQEGPFDVQET
EIAVQAKQPDVEEILSKGQHLYKEKPATQPVKRKLEDLSSEWKAVNRLLQELRAKQPDLAPGLTTIGASPTQTV
TLVTQPVVTKETAISKLEMPSSLMLEVPALADFNRAWTELTDWLSLLDQVIKSQRVMVGDLEDINEMIIKQKAT
MQDLEQRRPQLEELITAAQNLKNKTSNQEARTIITDRIERIQNQWDEVQEHLQNRRQQLNEMLKDSTQWLEAKE
EAEQVLGQARAKLESWKEGPYTVDAIQKKITETKQLAKDLRQWQTNVDVANDLALKLLRDYSADDTRKVHMITE
NINASWRSIHKRVSEREAALEETHRLLQQFPLDLEKFLAWLTEAETTANVLQDATRKERLLEDSKGVKELMKQW
QDLQGEIEAHTDVYHNLDENSQKILRSLEGSDDAVLLQRRLDNMNFKWSELRKKSLNIRSHLEASSDQWKRLHL
SLQELLVWLQLKDDELSRQAPIGGDFPAVQKQNDVHRAFKRELKTKEPVIMSTLETVRIFLTEQPLEGLEKLYQ
EPRELPPEERAQNVTRLLRKQAEEVNTEWEKLNLHSADWQRKIDETLERLQELQEATDELDLKLRQAEVIKGSW
QPVGDLLIDSLQDHLEKVKALRGEIAPLKENVSHVNDLARQLTTLGIQLSPYNLSTLEDLNTRWKLLQVAVEDR
VRQLHEAHRDFGPASQHFLSTSVQGPWERAISPNKVPYYINHETQTTCWDHPKMTELYQSLADLNNVRFSAYRT
AMKLRRLQKALCLDLLSLSAACDALDQHNLKQNDQPMDILQIINCLTTIYDRLEQEHNNLVNVPLCVDMCLNWL
LNVYDTGRTGRIRVLSFKTGIISLCKAHLEDKYRYLFKQVASSTGFCDQRRLGLLLHDSIQIPRQLGEVASFGG
SNIEPSVRSCFQFANNKPEIEAALFLDWMRLEPQSMVWLPVLHRVAAAETAKHQAKCNICKECPIIGFRYRSLK
HFNYDICQSCFFSGRVAKGHKMHYPMVEYCTPTTSGEDVRDFAKVLKNKFRTKRYFAKHPRMGYLPVQTVLEGD
NMETPVTLINFWPVDSAPASSPQLSHDDTHSRIEHYASRLAEMENSNGSYLNDSISPNESIDDEHLLIQHYCQS
LNQDSPLSQPRSPAQILISLESEERGELERILADLEEENRNLQAEYDRLKQQHEHKGLSPLPSPPEMMPTSPQS
PRDAELIAEAKLLRQHKGRLEARMQILEDHNKQLESQLHRLRQLLEQPQAEAKVNGTTVSSPSTSLQRSDSSQP
MLLRVVGSQTSDSMGEEDLLSPPQDTSTGLEEVMEQLNNSFPSSRGRNTPGKPMREDTM

6.0 References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention.

More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A composition comprising:

(a) a recombinant adeno-associated viral vector that comprises a nucleic acid segment that encodes a mammalian therapeutic agent; and

(b) a water-soluble biocompatible gel.

2. The composition of claim 1, wherein said biocompatible gel comprises a sol, a matrix, a biogel, a hydrogel, a polymer, a polysaccharide, an oligosaccharide, or a viscous suspension.

3. The composition of claim 1 or claim 2, wherein said biocompatible gel comprises a polymer, a viscosity agent or sucrose.

4. The composition of claim 3, wherein said biocompatible gel comprises iodixanol or sucrose acetate isobutyrate.

5. The composition of any preceding claim, wherein said biocompatible gel comprises glycerin, gelatin, or alginate.

6. The composition of any preceding claim, wherein said biocompatible gel comprises SAF-Gel, Duoderm Hydroactive Gel, Nu-Gel; Carrasyn (V) Acemannan Hydrogel, Elta Hydrogel or K-Y Sterile Gel.

7. The composition of any preceding claim, wherein said biocompatible gel comprises a cross-linked or a conjugated gel.

8. The composition of any preceding claim, wherein said biocompatible gel comprises a cross-linked or conjugated biodegradable gel or gel matrix.

9. The composition of any preceding claim, wherein said recombinant adeno-associated viral vector is present in said composition at a concentration of at least 1×10^{11} AAV particles per milliliter.

10. The composition of any preceding claim, wherein said recombinant adeno-associated viral vector is present in said composition at a concentration of at least 1×10^{12} AAV particles per milliliter.

11. The composition of any preceding claim, wherein said recombinant adeno-associated viral vector is present in said composition at a concentration of at least 1×10^{13} AAV particles per milliliter.

12. The composition of any preceding claim, wherein said biocompatible gel comprises at least about 65% by weight of said composition.

13. The composition of any preceding claim, wherein said biocompatible gel comprises at least about 75% by weight of said composition.

14. The composition of any preceding claim, wherein said biocompatible gel comprises at least about 85% by weight of said composition.

15. The composition of any preceding claim, wherein said biocompatible gel comprises at least about 95% by weight of said composition.

16. The composition of claim 1, wherein said mammalian therapeutic agent is a peptide, polypeptide, enzyme, protein, antisense, or ribozyme.

17. The composition of claim 1, wherein said mammalian therapeutic agent is a peptide, polypeptide, enzyme, protein, antisense, or ribozyme that can be expressed in human muscle tissue.

18. The composition of claim 17, wherein said mammalian therapeutic agent is a peptide, polypeptide, enzyme, protein, antisense, or ribozyme that can be expressed in human cardiac or diaphragm muscle tissue.

19. The composition of claim 18, wherein said mammalian therapeutic agent is a biologically-active acid α-glucosidase (GAA), dystrophin, or α-1 antitrypsin polypeptide.

20. The composition of any preceding claim, further comprising a pharmaceutical excipient, buffer, carrier, or diluent.

21. The composition of any preceding claim, for use in therapy.

22. The composition of any preceding claim, for use in therapy of a mammal.

23. The composition of any preceding claim, for use in therapy of a human.

24. The composition of any preceding claim, for use in therapy of a human having, suspected of having, or at risk for developing a musculoskeletal disorder or dysfunction, a glycogen storage disease, Pompe's Disease, or a congenital myopathy.

25. The composition of any preceding claim, for use in therapy of a human having, suspected of having, or at risk for developing Duchenne Becker muscular dystrophy, cardiac hypertrophy, or acid maltase deficiency (Pompe's Disease).

26. A kit for diagnosing, preventing, treating or ameliorating the symptoms of a diseases or disorder in a mammal comprising: (i) the composition of any one of claims 1 to 20; and (ii) instructions for using said kit.

27. Use of the composition of any one of claims 1 to 20, in the manufacture of a medicament for treating a disease, dysfunction, or disorder in a mammal.

28. Use according to claim 27, wherein said composition is provided to said mammal by injection, infection, direct, or indirect administration to a cell, tissue, or organ of said mammal.

29. Use according to claim 27 or claim 28, wherein said mammal is human.

30. Use according to any one of claims 27 to 29, wherein said mammal is a human that has, is suspected of having, or at risk for developing a musculoskeletal disorder or a congenital myopathy.

31. Use according to any one of claims 27 to 30, wherein said mammal is a human that has, is suspected of having, or at risk for developing muscular dystrophy.

32. A method of providing an effective amount of rAAV particles to a mammal in need thereof, said method comprising the step of providing to said mammal, the composition of any one of claims 1 to 20, in an amount and for a time effective to provide said therapeutically effective amount of said rAAV particles to said mammal.

33. The method according to claim 32, wherein said composition is provided to said mammal systemically, or by direct or indirect administration to a cell, tissue, or organ of said mammal.

34. The method according to claim 32 or claim 33, wherein said composition is provided to mammal by direct injection, topical application, or transdermal administration.

35. A method of treating a disease, dysfunction, or disorder in a mammal, said method comprising the step of providing to a mammal in need thereof, the composition of any one of claims 1 to 20, in an amount and for a time effective to treat said disease, dysfunction, or disorder in said mammal.

36. The method according to claim 35, wherein said composition is provided to said mammal systemically, or by direct or indirect administration to a cell, tissue, or organ of said mammal.

37. A method for treating or ameliorating the symptoms of a congenital myopathy in a mammal, said method comprising administering to said mammal the composition of any one of claims 1 to 20; in an amount and for a time sufficient to treat or ameliorate the symptoms of said congenital myopathy in said mammal.

38. The method according to claim 37, wherein said congenital myopathy is muscular dystrophy.

39. A method for providing a mammal in need thereof an effective amount of a therapeutic agent, said method comprising introducing into suitable cells or a tissue of said mammal, an amount of the composition of any one of claims 1 to 20, for a time effective to provide said mammal with said effective amount of said therapeutic agent.

40. The method according to claim 39, wherein said therapeutic agent is a therapeutic peptide, polypeptide, protein, antibody, antisense polynucleotide, antisense oligonucleotide, catalytic ribozyme or an RNA molecule.

41. The method according to claim 39 or claim 40, wherein said composition is introduced into said cells or said tissue by systemic or localized injection, or by intramuscular, subcutaneous, intraabdominal, topical, or transdermal administration.

42. The method according to any one of claims 39 to 41, wherein said composition is introduced into said cells or said tissue by transfection.

43. The method according to any one of claims 39 to 42, wherein said composition is introduced into said cells or said tissue by direct injection into a muscle of said mammal.

44. The method according to any one of claims 39 to 43, wherein said composition is introduced into said cells or said tissue by direct injection into the diaphragm or cardiac muscle of said mammal.

45. The method according to any one of claims 39 to 44, wherein said therapeutic polypeptide is an enzyme, a kinase, a protease, a glucosidase, a glycosidase, a nuclease, a growth factor, a tissue factor, a myogenic factor, a neurotrophin, a dystrophin, an interleukin, or a cytokine.

46. The method according to any one of claims 39 to 45, wherein said therapeutic polypeptide is acid α-glucosidase (GAA).

47. The method according to any one of claims 39 to 46, wherein said composition is introduced into said population of said mammalian diaphragm, heart, or muscle cells by systemic, indirect, or localized infection, or by intramuscular, subcutaneous, intra-abdominal, transpleural, intracardiac, or transperitoneal injection.

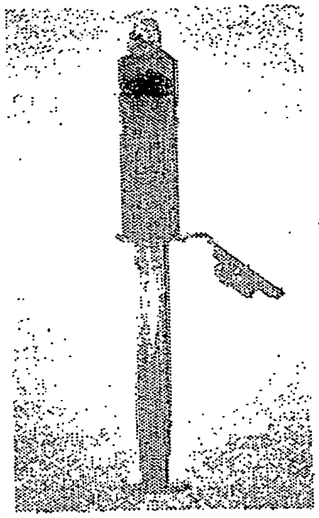

FIG. 1C

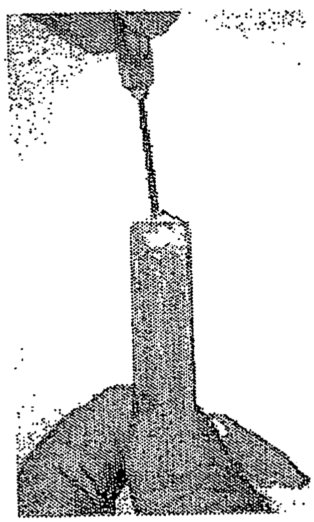

FIG. 1B

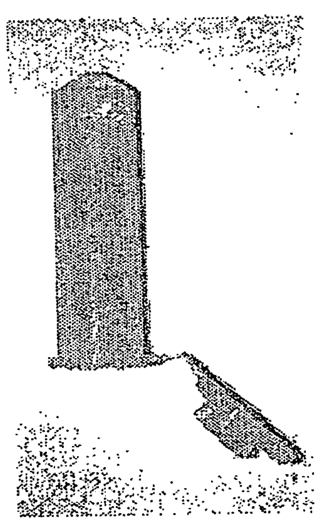

FIG. 1A

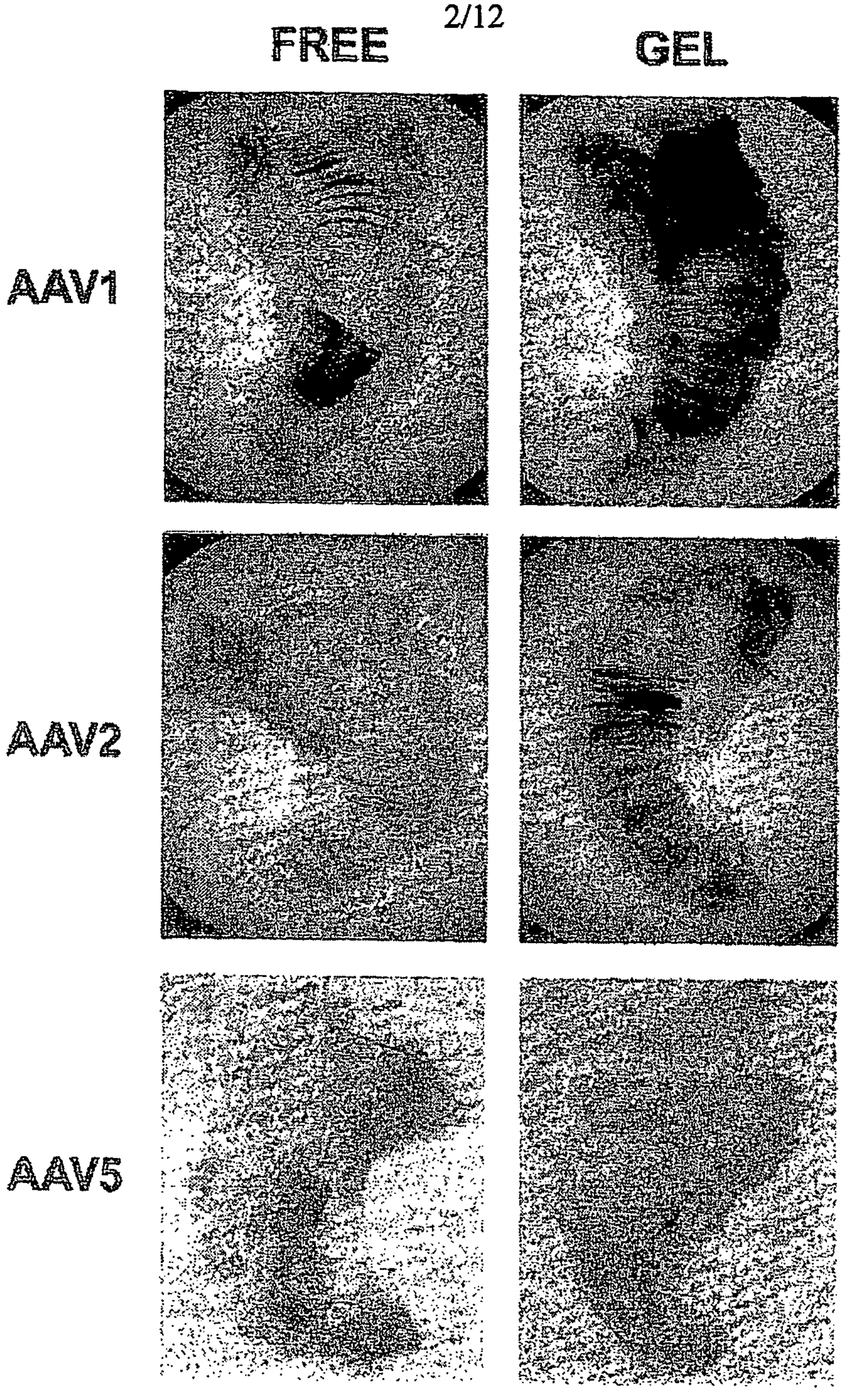

FIG. 2A

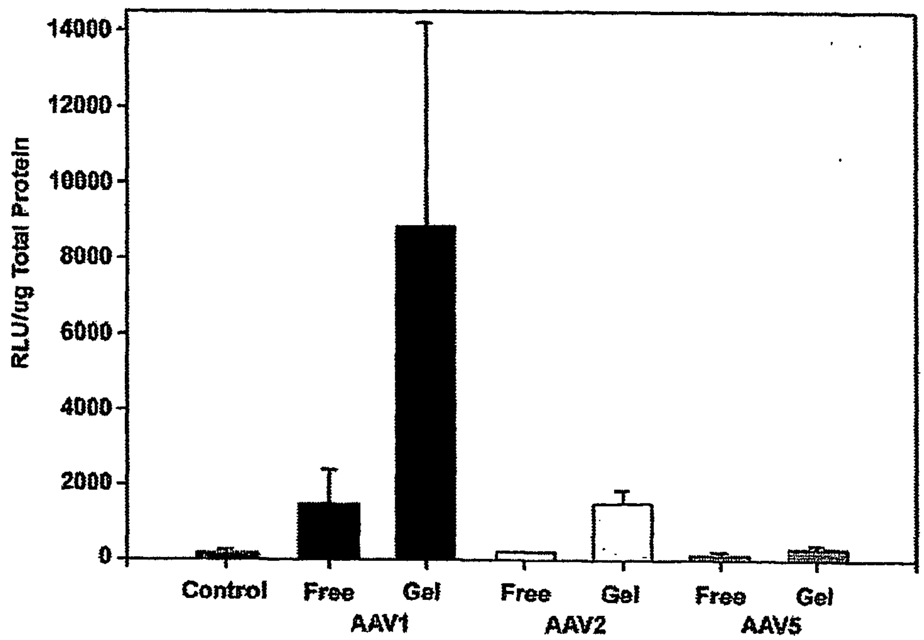
RLU/ug Total Protein
14000
12000
10000
8000
6000
4000
2000
0
Control
Free
Gel
AAV1
Free
Gel
AAV2
Free
Gel
AAV5

FIG. 2B

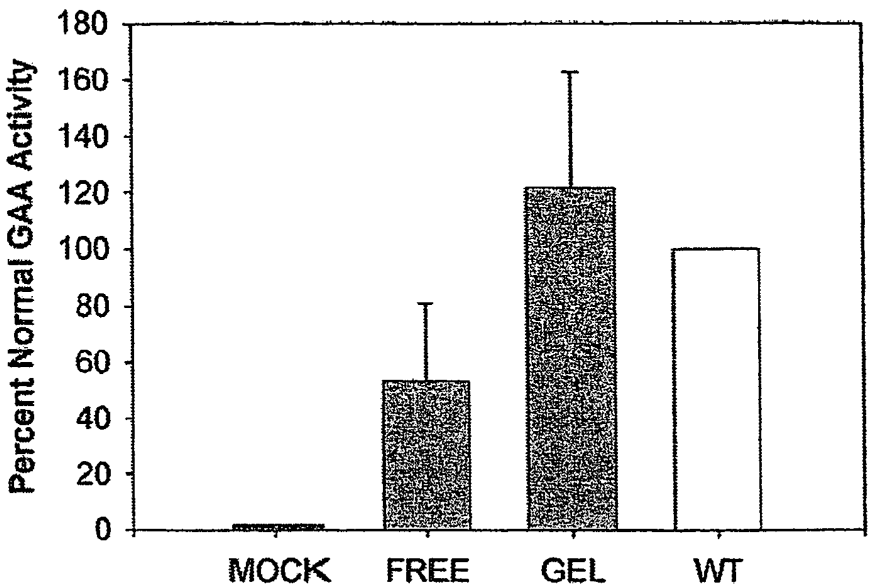
180
160
140
120
100
80
60
40
20
0
Percent Normal GAA Activity
MOCK
FREE
GEL
WT

FIG. 3A

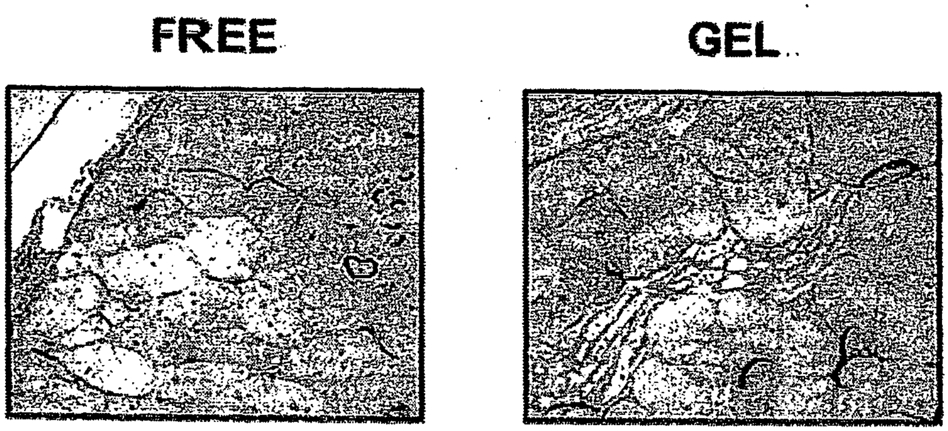

FIG. 3B

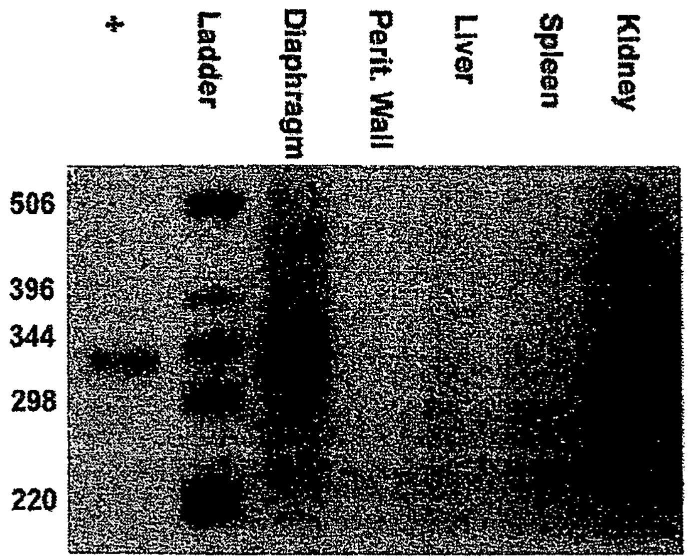
+
Ladder
Diaphragm
Perit. Wall
Liver
Spleen
Kidney
506
396
344
298
220

FIG. 4

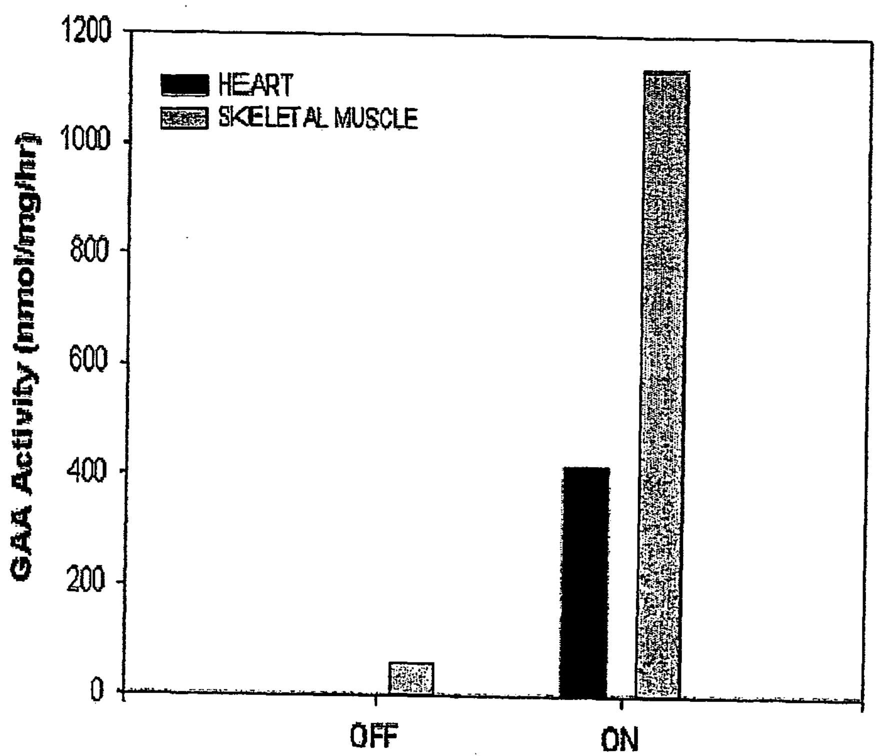
1200
1000
800
600
400
200
0
GAA Activity (nmol/mg/hr)
HEART
SKELETAL MUSCLE
OFF
ON

FIG. 5

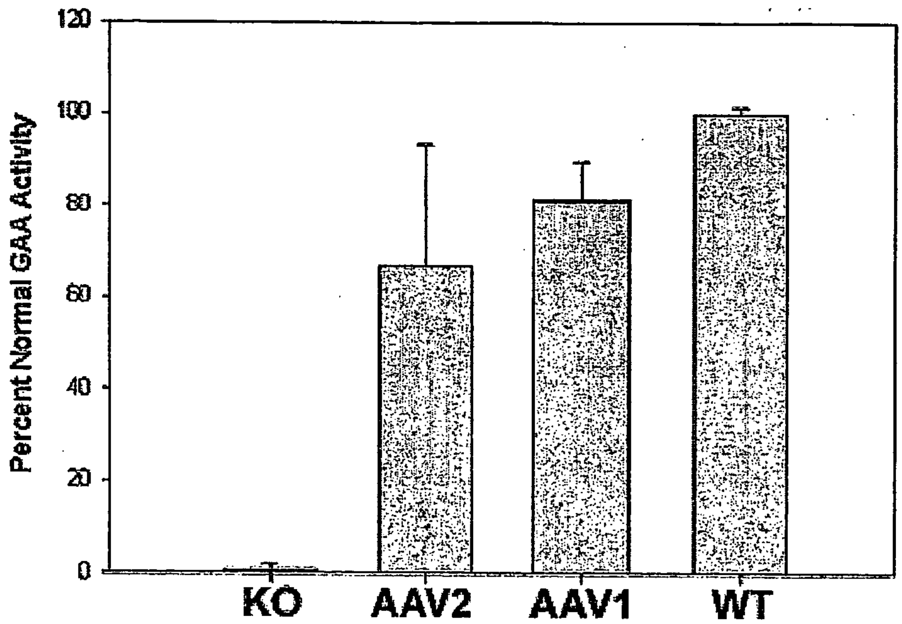
120
100
80
60
40
20
0
Percent Normal GAA Activity
KO
AAV2
AAV1
WT

FIG. 6

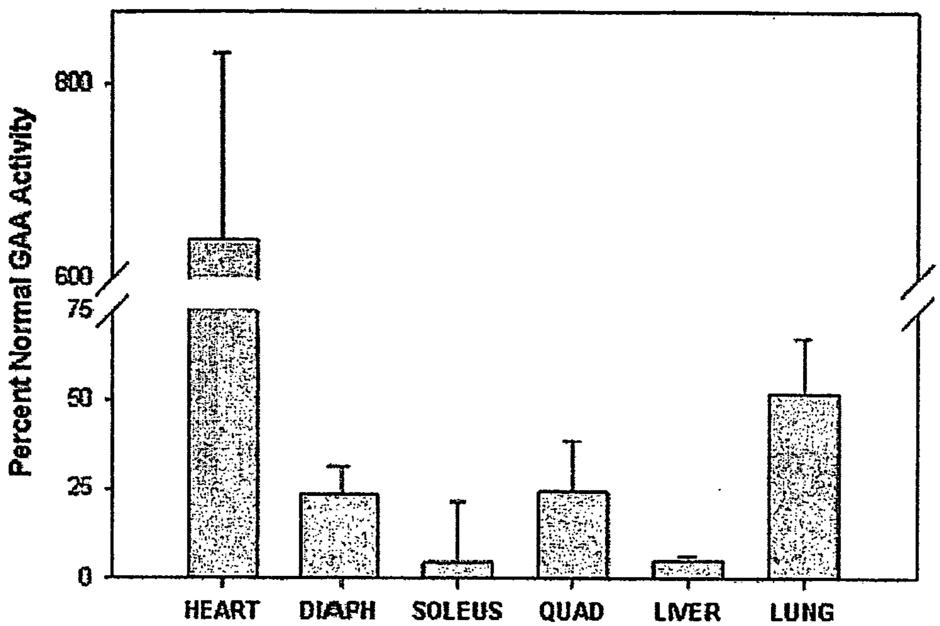
Percent Normal GAA Activity
800
600
75
50
25
0
HEART
DIAPH
SOLEUS
QUAD
LIVER
LUNG

FIG. 7

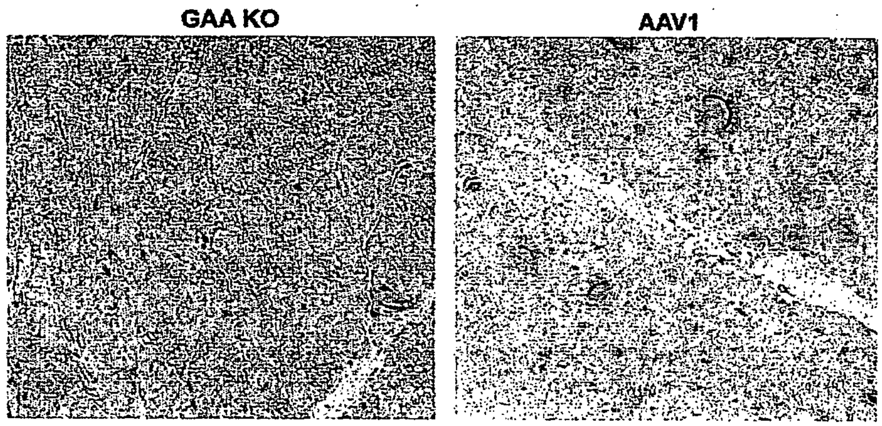

FIG. 8

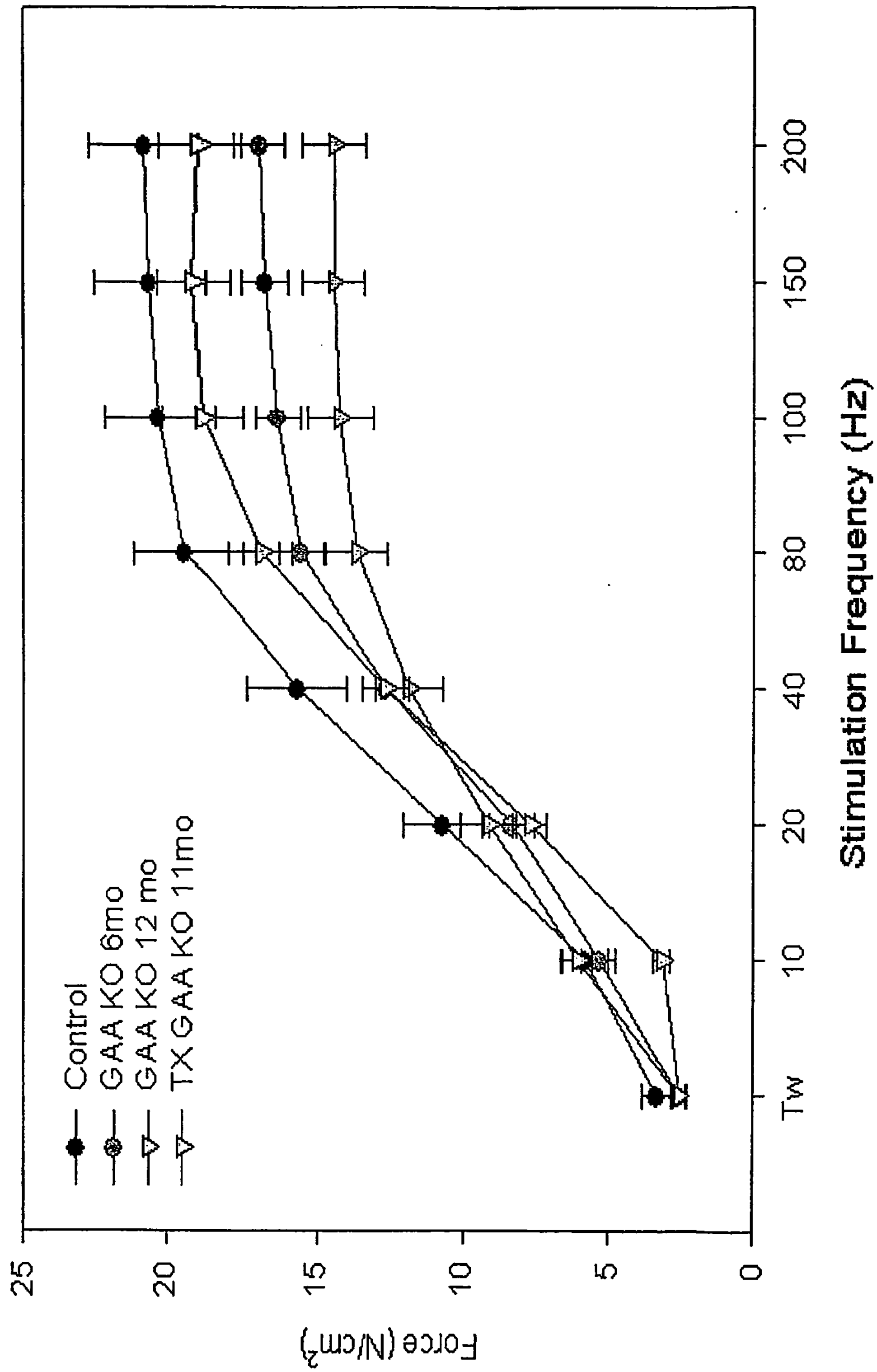
Control
GAA KO 6mo
GAA KO 12 mo
TX GAA KO 11mo
Force (N/cm²)
0
5
10
15
20
25
Tw
10
20
40
80
100
150
200
Stimulation Frequency (Hz)

FIG. 9

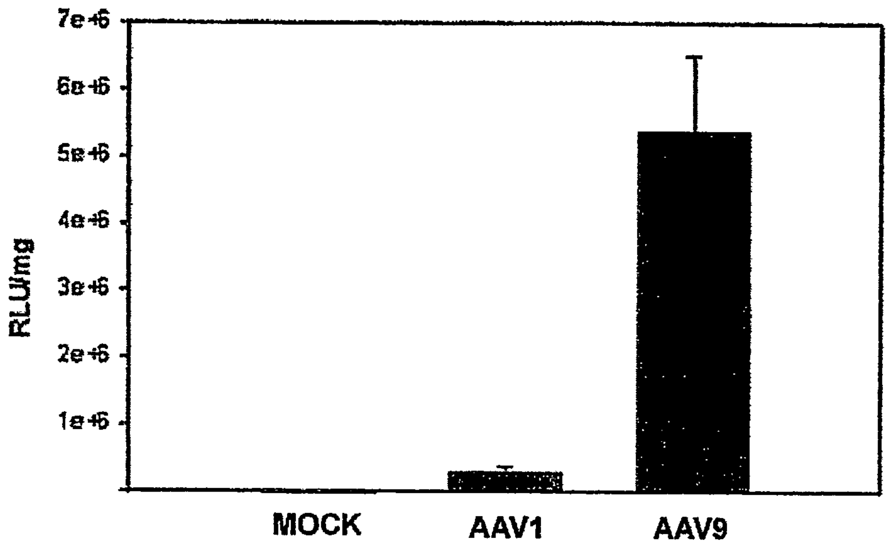

FIG. 10